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EXPERIMENTAL CHEMOTHERAPY OF FILARIASIS

I. Experimental Methods for Testing Drugs Against Naturally Acquired Filarial Infections in Cotton Rats and Dogs

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THE exposure of our armed personnel to filariasis in endemic regions of the Pacific theater of war stimulated the search for new filaricidal drugs in this country. In 1945 a report on the known methods of treatment for filariasis was prepared by Temkin for the Division of Medical Sciences of the National Research Council.[1] In this report are listed the results obtained by various investigators, chiefly with derivatives of heavy metals, in their attempts to cure the disease in man.

Recent reports by Brown[2] and by Culbertson, Rose, and Oliver-Gonzalez[3-5] demonstrate promising results in man by the use of Lithium Antimony Thiomalate and Neostibosan.

Until very recently the search for new drugs to treat filariasis has been limited because of the lack of a small, easily available laboratory host. A large proportion of the early experimental chemotherapy on this disease was carried out in dogs infected with *Dirofilaria immitis*.[6] The routine screening of new compounds in the dog, however, is virtually impossible because of the relatively large amounts of drug needed to treat a single animal. Various investigators have reported the successful treatment of Dirofilaria infections with antimonials, and Fuadin[7, 8] is at present the drug of choice for the treatment of heartworm infections in dogs. Otto and Maren[9] have reported the use of a new arsenical for treating this infection.

Litomosoides carinii, a filarial parasite of the cotton rat (*Sigmodon hispidus*), is now being used in many laboratories for the bio-assay of filaricidal drugs.[10-13] The vector of this parasite has been found to be the tropical rat mite (*Liponyssus bacoti*)[14, 15] and several papers have been published regarding the host-parasite relationships.[16-19] Some information has been made available regarding the effects of chemical compounds other than antimonials against this infection,[10, 11, 23] chiefly the cyanine dyes.

Since April, 1945, filaria-infected cotton rats have been used in these laboratories for the routine screening of various nonmetallic, organic compounds for filaricidal properties. The methods used for bio assay, both in cotton rats and dogs, and the results of tests on compounds in cotton rats are reported herein.

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EXPERIMENTAL METHODS IN COTTON RATS

Naturally infected cotton rats* were used as experimental hosts. Animals of various ages and both sexes were used. The number of microfilariae present in the blood stream upon arrival varied considerably, and no method was devised for determining the number of adult worms present in the pleural cavity before treatment was initiated.

Bell and Brown[18] have demonstrated that the microfilariae of *Litomosoides carinii* do not exhibit nocturnal or diurnal periodicity, and our studies confirm these observations (Table II).

Method of Counting Microfilariae.—The first criterion which was used for determining the effect of a new drug against this infection was the behavior of the microfilarial count in the peripheral blood. Rather than strive for absolute, quantitative values it was apparent that much time would be saved by developing a quick qualitative method for examining the blood. Thin smears were made from one drop of blood obtained from the cut tails or toes of the rats. These were air dried and stained with Giemsa's stain, with or without dehemoglobinization. The presence of intact red cells did not materially detract from the ease with which embryos could be found under a low-power objective of the microscope. Generally, from 25 to 100 microscopic fields were counted, but where very few microfilariae were present, 160 fields were always counted. The count from all animals was then expressed per 100 microscopic fields.

It is recognized that this method is subject to considerable error, but our interest was primarily in an all-or-none effect, and all animals under treatment were handled in the same way.

In our first experimental series, microfilarial counts were made on the day before treatment was initiated and every day thereafter for from two to four weeks. In later series this was considered unnecessary, and counts were made only before treatment and on the day of autopsy.

Microfilarial Counts in Untreated Rats.—In Tables I, II, and III data are given from untreated rats for varying periods of observation. In each case it will be noted that considerable fluctuation occurred in microfilarial counts, regardless of how often slides were examined.

The weekly records (Table III) provide the most pertinent information. It will be noted that all of the rats showed a marked increase in microfilariae over a period of several weeks observation. This denotes that the animals were probably infected with worms which had not all reached maturity when received and subsequently discharged increasingly larger numbers of embryos. Similar observations have been made in human infections in children.[3] The significance of this observation is that decreases in microfilariae which occur in treated animals are probably greater than actually observed over a period of time.

In only one untreated rat of sixty-five which have been studied has the microfilarial count dropped spontaneously to zero.

Description of Adult Worms in Cotton Rats.—Litomosoides carinii is a parasite of the pleural cavity in cotton rats and with few exceptions in more

*Obtained from the Hegener Research Supply Company, Sarasota, Fla.

TABLE I. UNTREATED COTTON RATS—MICROFILARIAL COUNTS ON ALTERNATE DAYS

RAT	MICROFILARIAE PER 100 LOW-POWER FIELDS																			
	DAY																			
	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35		
41	244	156	240	112	316	675	248	308	268	180	336	128	396	152	368	164	164	196		
108	252	372	144	360	248	120	64	56	204	30	252	312	428	104	88	168	80	220		
125	204	336	212	244	576	244	192	328	268	124	296	92	308	212	260	224	272	452		
182	272	328	236	204	188	184	120	192	184	576	304	272	552	344	412	610				
200	34	56	64	32	34	30	44	24	24	44	52	60	96	56	7	46				
193	64	44	40	48	12	32	56	24	28	52	52	64	8	68	52	124				
195	116	60	108	112	108	104	64	60	68	196	64	44	140	184	72	244				
198	176	88	372	120	140	260	212	92	360	160	160	268	324	472	268	244				

TABLE II. UNTREATED COTTON RATS—MICROFILARIAL COUNTS EVERY TWO HOURS

RAT	MICROFILARIAE PER 100 LOW-POWER FIELDS																							
	HOUR																							
	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
227	32	14	30	18	6	10	16	12	14	14	30	14	44	44	20	60	30	56	88	76	56	60	40	108	44
241	68	32	36	4	44	40	104	96	88	44	18	48	28	26	128	100	84	172	184	144	180	152	116	76	96
247	60	24	16	96	18	10	56	52	42	8	32	40	28	16	76	54	40	56	60	76	26	*			
249	16	18	16	4	12	0	20	4	16	20	24	10	6	8	16	20	14	12	30	30	20	40	20	60	8

*, Death.

TABLE III. UNTREATED COTTON RATS—WEEKLY MICROFILARIAL COUNTS

	MICROFILARIAE PER 100 LOW-POWER FIELDS																	
	WEEK																	
RAT	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
150	75	88	176	420	220	540	420	332	220	212	1,152	392	792	204	256	*		
151	34	22	56	160	232	460	216	400	920	552	596	536	668	288	480	648	1,004	1,070
153	56	168	276	332	398	700	332	948	*									
154	42	84	44	132	76	260	260	356	296	380	536	940	–	–	508	520	596	440
155	40	24	124	128	108	312	160	212	*									
156	116	88	300	396	264	580	176	604	*									
157	32	60	152	240	432	352	372	496	*									
158	40	148	26	68	40	372	268	308	*									
41	244	112	308	400	152	164	140	184	168	132	236	228	264	116	256	332		
108	252	360	56	142	104	564	112	152	312	200	76	92	176	248	140	312		
125	204	244	328	188	212	388	344	352	632	452	248	216	480	388	380	364		
182	200	204	120	304	344	400	652	364	816	500	528	652						
200	44	32	44	52	132	92	56	116	120	80	80							
193	56	48	24	52	68	–	44											
195	72	112	60	64	184	–	136	416	384	140	120	368	400					
198	212	120	92	160	472	–	600	284	632	236	592	520	1480					
201	20	22	18	8	0	–	*											
227	32	18	16	–	104	64	44	72	76	56								
241	68	16	84	–	208	100	144	200	164	168								
249	16	12	22	–	72	60	28	44	34	48								
362	40	56	68	14														
368	40	56	20	12														
388	560	410	240	720														
389	120	780	430	88														

–, No reading taken.
*, Death.

TABLE IV. UNTREATED COTTON RATS—PER CENT DIFFERENCE FROM ORIGINAL MICROFILARIAL COUNT AT END OF OBSERVATION PERIOD

RAT	NUMBER WEEKS OBSERVED	PER CENT DIFFERENCE FROM ORIGINAL COUNT	
		INCREASE	DECREASE
150	14	254	
151	17	3,047	
153	7	1,593	
154	17	947	
155	7	430	
156	7	420	
157	7	1,450	
158	7	670	
41	15	36	
108	15	23	
125	15	78	
182	11	246	
200	11	81	
193	6		22
195	12	455	
198	12	598	
201	4		100
227	9	75	
241	9	147	
249	9	200	
362	3		65
368	3		70
388	3	28	
389	3		27

than 1,000 autopsies adult worms have never been found in any other site. In a few cases no worms were found in the pleural cavity, but several occurred free in the abdominal cavity. When many worms were present they occurred intertwined in masses, generally applied to the dorsal wall, or were located in the pockets formed between the lobes of the lungs and the diaphragm. Frequently, they were applied to the pericardial membrane or to the outer surface of the lungs. Macroscopic host tissue reactions to the presence of living worms rarely were found, and when they were removed the pleural walls appeared shiny, glistening, and apparently undamaged.

Living worms recovered from the pleural cavity invariably undergo writhing movement when placed in 0.8 per cent saline solution and continue this activity for several hours at room temperature. They are slick, shiny, and transparent when viewed under a binocular dissecting microscope, and the internal structure is clearly defined.

In some animals in which dead worms were found at autopsy, particularly those in which large numbers of worms were killed, extensive adhesions of the heart and lungs to the pleural cavity wall frequently occurred. Isolated dead worms showed no movement whatever when placed in 0.8 per cent saline and were stretched out for their full length. When viewed under a microscope they had a dull, opaque appearance, and the internal structure was not clearly defined.

When dead worms occurred in masses they were sometimes, but not always, encased in a caseous, purulent "exudate." Culbertson and Rose[12] have described a similar substance surrounding dead worms following treatment with antimony derivatives. Bertram, Unsworth, and Gordon[16] have also described this substance surrounding dead worms in white albino rats which were

experimentally infected with *Litomosoides carinii* through the use of the vector *Liponyssus bacoti*. We have found it to consist largely of leucocytes and fat and believe that it represents a response of the host to the presence of decaying worms.

In later stages of decay, dead worms occurred in tight, shrunken masses, were yellowish in color, and were fragmented.

Of sixty-five untreated rats which were subjected to autopsy, nine showed a small number of dead worms. In no case, however, were large numbers of dead worms present, and caseous or purulent material was rarely found.

Methods of Dosage and Evaluation of Drugs in Cotton Rats.—In common with all host-parasite relationships used for evaluating the effectiveness of drugs, several different methods are available to the investigator for determining whether new chemical compounds are active against filariasis in cotton rats. The amount of drug given, route of administration, number of days of treatment, frequency of treatment, and time of autopsy are all variables to which some degree of standardization must be assigned if comparisons are to be made with known filaricidal substances.

Using cotton rats as experimental hosts, the only published reports in detail relative to the effectiveness of treatment with antimony compounds are those of Culbertson and Rose,[12] Culbertson and Price,[13] and Lawton and coworkers.[20] The effect of cyanine dyes against filariasis in cotton rats has been presented recently.[10, 11, 23] Culbertson and Rose demonstrated that large intramuscular doses of Neostam and Neostibosan (400 to 800 mg. per kilogram) administered once daily, with varying intervals between doses, killed the adult worms and produced a gradual disappearance of microfilariae from the peripheral blood. Essentially the same results were obtained by Culbertson and Price[13] with Stibanose (Solustibosan). We have been able to confirm these results in a small series of cotton rats.

Due to the paucity of information regarding the effectiveness of compounds other than antimonials against filariasis in any vertebrate host, we believed it important to study effects produced by new compounds against both microfilariae and macrofilariae in our screening tests.

In our program, compounds received for testing were administered orally or intraperitoneally to cotton rats, depending upon their solubility. If a sufficient quantity of the drug was available, 100 to 200 mg. per kilogram were given orally, or 25 to 50 mg. per kilogram intraperitoneally, as a starting dose. Previous experience with drugs used for screening purposes in other host-parasite relationships frequently determined the starting dose. If the animals survived, treatment was administered twice daily for from two to four weeks. For preliminary screening, from one to three animals were used for each test. If the compound proved toxic, and a sufficient supply was available, the dose was halved and retested. Active compounds were tested at many dose ranges.

Insoluble drugs were suspended in a 2.5 per cent solution of corn starch and were administered orally through a 2½ inch, 18-gauge hypodermic needle with a blunted end. Soluble drugs were dissolved in water and were administered intraperitoneally through a ½ inch, 23-gauge hypodermic needle.

In general, autopsies were performed at intervals of from two to four weeks after cessation of treatment, although this procedure was not strictly adhered to in the case of compounds showing suggestive activity in previous tests. Large groups of animals were used when compounds showed an effect against either microfilariae or macrofilariae, and autopsies were performed at various intervals after cessation of treatment. In cases where large masses of living or dead worms were present no attempt was made to count the exact number.

When precipitous and sustained reductions in microfilariae occurred during treatment, or dead adult worms were found at autopsy following treatment, repeated tests were made on compounds producing these effects. Animals with low initial microfilaria counts (namely, less than 10 per 100 fields) were found to be very unsatisfactory for assay purposes, since reductions in microfilariae which might occur were well within the limits of nontreated controls.

Certain obvious sources of error are apparent in the procedures described for the routine screening of compounds against this infection. Longer periods of treatment, more frequent treatment, or longer intervals between cessation of treatment and autopsy might have revealed more active compounds than have as yet been found. Since practically nothing was known about the absorption, excretion, or toxicity of the compounds before tests were made, however, it became necessary to adopt some uniform plan and to treat all compounds alike until experience demonstrated that other methods would produce better results. Similar situations have been experienced in all screening programs, using various host-parasite relationships. Very little is known concerning the mode of action of known filaricidal drugs, and it seemed to us that the trial-and-error method of bio-assay in searching for new drugs was the best method of approach.

EXPERIMENTAL METHODS IN DOGS

Adult *Dirofilaria immitis* commonly occur in the right ventricle of the heart or may be found in the pulmonary artery near the heart. The number of adult worms probably determines the severity of symptoms; namely, emaciation, weakness, jaundice, and a persistent cough. Worms which may be killed by drugs, and probably some which die naturally, are found in the terminal branches of the pulmonary artery within the lung. This has been described by Wright and Underwood[7] in dogs treated with Fuadin.

If a large number of worms are present in the heart when treatment with an active compound is begun, it has been assumed, and with some good substantiating evidence, that worms which are killed quickly and simultaneously will produce an embolus in the pulmonary artery and kill the dog. Heavily infected dogs, namely, those which show a high microfilarial count with severe symptoms, are therefore considered poor risks for treatment.

In our experiments dogs have been used only for determining the effectiveness of compounds which showed activity in the cotton rat. Essentially the same procedures for treatment were used as described for the cotton rat, and previous results from the rats provided a rough estimate of the probable tolerated dosage in dogs. Oral doses were administered in capsules.

Microfilarial counts from dogs were made by withdrawing 0.05 c.c. of blood from the large vein in one of the forelegs, placing a 22 by 40 mm. cover slip over the blood on a glass slide, and counting the entire fresh preparation. Quantitative methods were more feasible in the dogs than in cotton rats, since fewer animals were used, and measured samples of blood could be withdrawn frequently without seriously disturbing the host.

In some untreated dogs, as well as treated dogs with initial low embryo counts (from 2 to 10 microfilariae per 0.05 c.c.), no adult worms were found at autopsy. This has been experienced by other investigators.

As has been mentioned for cotton rats, dogs with low initial microfilarial counts are unsatisfactory for assay purposes. Any decrease which might occur in the microfilarial count in such animals following treatment cannot be measured either qualitatively or quantitatively. Moreover, a large percentage of these cases invariably reveal no adult worms at autopsy, and the results of treatment are therefore exceedingly questionable.

Following treatment with Fuadin, the microfilarial count is reduced within one to several weeks and varying numbers of dead worms are found in the lungs at autopsy.[7, 8] It is frequently necessary to push the dosage to the limit of tolerance, however, and in some cases death occurs from acute toxicity.

For determining the effectiveness of new compounds against Dirofilaria infections in dogs, we have used three criteria: (1) a rapid and sustained reduction in microfilariae, (2) presence of dead worms in the terminal blood vessels within the lung at autopsy, and (3) improved physical condition of the animal. The improvement in the physical conditions of animals following treatment is not a certain sign that cure has been affected if it is not accompanied by the destruction of the worms. We have frequently received dogs for treatment which were emaciated, sickly, and unkempt. Some of these animals were caged and fed adequately for a number of weeks preceding treatment, were freed of fleas, and were cured of mange, and all improved in appearance markedly during their pretreatment period, in spite of the fact that a relatively large number of microfilariae were present in the peripheral blood.

COMPOUNDS TESTED FOR FILARICIDAL ACTIVITY IN COTTON RATS

In our screening program, 517 organic, nonmetallic compounds have been tested for activity against *Litomosoides carinii* in the cotton rat. These included 78 organic dyes (phenazines, thiazines, oxazines, triphenylmethanes, phenoxazines, acridines, anthraquinones, and fluorenes); 31 acids and derivatives, 21 aromatic amines, 13 aromatic ketones, 7 amino Alkanols, 8 methanes; 16 oxazolidines, 62 guanidines, melamines, biguanides, and guanyls, 112 piperazines, 15 pyridines, 8 metanilamides, 20 sulfonamides, 9 ureas, thioureas, and urethrones, 22 sulfides, sulfones, and sulfonic acids, 16 pyrimidines and pyrazines, 22 piperidines, 4 triazines, 5 quinolines and isoquinolines, 7 cyanamides and dicyandiamides, 6 imidazoles and pyrazoles, 8 dioxanes and morpholines, and 25 miscellaneous compounds, including antibiotics and plant extracts.

Several types of compounds showed activity in the cotton rat, either against microfilariae or macrofilariae or both. The best balance between activity and

toxicity, however, was found in the piperazines. The first piperazine tested which showed filaricidal activity was 1-carbethoxy-4-methylpiperazine hydrochloride (180-C). By the oral or intraperitoneal administration of doses ranging from 12.5 to 400 mg. per kilogram, this compound produced a rapid reduction of microfilariae in the peripheral blood and showed suggestive activity against the adult worms. Because of the unique effects produced by this compound and related piperazines, detailed results are presented in separate papers.[21, 22]

Data concerning active compounds other than piperazines will be presented when more complete information has been obtained.

DISCUSSION

The evaluation of new chemical compounds for therapeutic effectiveness against any naturally acquired infection in animals is subject to considerably more error in the interpretation of results than is the case where standardized infections are produced by introducing known numbers of parasites into laboratory hosts. In the case of such highly standardized infections, for example, as *Plasmodium lophurae* in the duck and *Trypanosoma equiperdum* in mice, a single screening test will often give definite information regarding the effect of a new compound. Moreover, in both of these laboratory infections, several known compounds of widely different chemical structure can be used as control drugs.

For testing new compounds for filaricidal properties, no standardized laboratory infection has as yet been devised. The vectors of both *Litomosoides carinii* in the cotton rat[14, 15] and *Dirofilaria immitis* in the dog are known, and it is quite probable that in the future a method will be available for testing drugs against experimental infections of known character in these animals. The use of *Dirofilaria immitis* in the dog, however, will probably never prove satisfactory for a large scale screening programs, due to the obvious disadvantages of a large experimental host.

In evaluating the effects of the different types compounds tested in these laboratories, an attempt was made in each case to determine the effect produced upon both microfilariae and macrofilariae in the cotton rat. Sometimes this was not possible, since the animals died while under treatment and the supply of drug was not sufficient to make a second trial. In such cases, when no reduction in microfilariae occurred during treatment and the animals died before the time planned for autopsy, the drug was listed as inactive. Obviously, this cannot be considered a true appraisal of the drug in every case, since compounds are known which produce no direct effect upon the microfilariae but do kill the adult worms.[12, 13]

The time which elapses between cessation of treatment and autopsy is also a factor which may influence the evaluation of new compounds, as will be demonstrated in subsequent papers.[21, 22]

Nontreated control animals and animals treated with filaricidal antimony compounds were not included in each protocol in our screening program. The effects of antimony derivatives against *Litomosoides carinii* have been substan-

tially demonstrated[12, 13] and the course of untreated infections was studied at various times. Since the animals received varied in age, weight, and duration and intensity of infection, and because there was no way for determining how many adult worms were present, any suggestion of activity produced by new compounds was followed by repeated tests.

Recognizing the variables which accompany the use of naturally infected cotton rats for screening new compounds against filariasis, we believe that this host-parasite relationship provides a useful tool in the search for new filaricides, particularly since no other small host is available which will serve the purpose. The correlation between activity in cotton rats, dogs, and human beings infected with filaria has yet to be determined.

SUMMARY

Methods used in testing new chemical compounds for filaricidal activity in naturally infected cotton rats and dogs are described. Data are given relative to untreated infections in cotton rats, and the types of compounds tested are mentioned. The filaricidal effect of 1-carbethoxy-4-methylpiperazine hydrochloride is discussed.

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EXPERIMENTAL CHEMOTHERAPY OF FILARIASIS

II. Effect of Piperazine Derivatives Against Naturally Acquired Filarial Infections in Cotton Rats and Dogs

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INTRODUCTION

IN THE preceding paper[1] it was mentioned that compound 180-C (1-carbethoxy-4-methylpiperazine hydrochloride) produced a reduction in microfilariae when administered orally or intraperitoneally to filaria-infected cotton rats, and that suggestive action was produced against the adult worms. The effect of this compound, on the microfilariae at least, was so much more rapid than had been reported for any other known compound that an investigation of other derivatives was indicated. The organic chemistry departments of the Lederle Laboratories Division and the Calco Chemical Division of American Cyanamid Company therefore prepared a number of piperazine compounds for testing in the cotton rat. The results of these tests are given in this paper, and the synthetic part of the program is reported elsewhere.[2, 3]

EXPERIMENTAL METHODS

In addition to the general procedures reported in a previous paper[1] for evaluating the effects of new compounds against filariasis in the cotton rat, supplementary methods were used in the case of the piperazines. Several members of this group of compounds were found to produce precipitous reductions in the microfilarial count following various oral or intraperitoneal doses. For comparative evaluations it was necessary therefore to select what appeared to be the most active derivative and to use this compound as a standard, particularly since no other known filaricidal compounds produced comparable effects. During the first few months of this study, 1-carbethoxy-4-methylpiperazine hydrochloride (compound 180-C) produced the most consistent effect against microfilariae and was used as a standard. Data from eighty-one cotton rats treated with various doses of 180-C are given in Table IV.

Although compound 180-C was markedly effective against microfilariae in well-tolerated doses in the cotton rat, the results obtained against adult worms (Table V), although suggestive, were not promising. Moreover, doses which reduced the microfilariae of *Dirofilaria immitis* in the dog frequently caused nausea, muscular weakness, profuse salivation, and prostration. Another compound was subsequently chosen as a standard. This was 1-diethylcarbamyl-4-methylpiperazine hydrochloride (84-L). Marked reductions in microfilariae were produced by this compound in doses as low as 3 mg. per kilogram in cotton rats; no serious toxic symptoms occurred in dogs in therapeutic doses; and by frequent administration a large proportion of the adult worms were killed in cotton rats. A detailed report on the effects produced by 84-L in cotton rats and dogs is given in another paper in this series,[4] but for comparative purposes some data regarding its activity are included herein.

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In testing new piperazine derivatives three rats were used for a preliminary assay. If the compound was soluble, intraperitoneal doses of 50, 25, and 12.5 mg. per kilogram were administered twice daily for from two to four weeks. Insoluble derivatives were administered orally in a 2.5 per cent starch suspension at 200, 100, and 50 mg. per kilogram. If preliminary assays proved the compounds to be toxic in the doses used, second or third trials were made, halving the doses each time. Autopsies were performed within one to four weeks after cessation of treatment.

In the case of piperazines which showed activity against microfilariae several dosage regimes were used and an attempt was made to determine an approximate chemotherapeutic index (Table II).

In several cases, due to a difficult synthesis, relatively small amounts of compounds were available. If these showed no activity in doses which were two or three times greater than the minimum effective dose of 84-L no further tests were performed. It is quite possible, therefore, that some of the compounds listed as inactive in Table I might have shown activity at higher doses.

Types of Piperazines Tested.—In Tables I and II are listed most of the piperazines and related compounds which have been tested thus far in cotton rats. Several other piperazines, some of which were active, will be reported in a later publication. The piperazine nucleus itself showed no activity nor did any of its components. The addition of the carbethoxy radical in position 1, with various substitutions in position 4, produced a number of compounds with high microfilaricidal activity. Alkyl radicals in position 4 retained high activity as far as the propyl and isopropyl derivatives, but butyl substitutions were much less active. Toxicity was increased as the alkyl chain was lengthened.

Retaining the carbethoxy group in position 1, two other substitutions in position 4 retained activity, namely guanyl and carbethoxy.

The only compounds lacking the carbethoxy group which showed marked activity against microfilariae were 1-ethylcarbamyl-4-methylpiperazine hydrochloride (182-L); 1-diisopropylcarbamyl-4-methylpiperazine hydrochloride (177-L); 1-dimethylcarbamyl-4-methylpiperazine hydrochloride (152-L); and 1-Diethylcarbamyl-4-methylpiperazine hydrochloride (84-L). The latter two compounds are considered as the most active of the piperazines yet tested.

Effect of Piperazines on Microfilariae in the Cotton Rat.—The piperazines listed in Tables II and III produced rapid reductions in microfilariae in the peripheral blood of cotton rats. In some cases the amount of drug necessary to produce this effect was very close to the lethal dose, whereas in others it was far beneath the lethal dose. In general active piperazines in proper doses produced a measurable reduction in microfilariae within twenty-four hours (Table III) after the first dose was administered, and this reduction was sustained for as long as treatment was continued. Recurrences of microfilariae frequently occurred after cessation of treatment.

An exhaustive study of all active piperazines has not been made thus far, since the number of animals and amount of drug necessary to carry out such a study would have been prohibitive, from the standpoint of both time and economy. The comparison given in Tables II and III, however, illustrate the effects of eighteen active piperazines, and Table IV illustrates the effects of different dosage upon microfilariae, using 1-carbethoxy-4-methylpiperazine hydrochloride.

TABLE I. PIPERAZINE AND RELATED COMPOUNDS SHOWING NO FILARICIDAL ACTIVITY IN COTTON RATS IN THE DOSES USED

COMPOUND	NAME	HIGHEST* DOSAGE USED (MG./KG.)
148-L	Ethyl carbamate	100 I.P.
150-L	Ethyl N-methylcarbamate	100 I.P.
149-L	Ethyl N-ethylcarbamate	100 I.P.
146-L	Ethyl N,N-diethylcarbamate	100 I.P.
144-L	N-carbethoxyethylenediamine	100 I.P.
220-C	Ethyl N-ethyl-N-(2-hydroxyethyl)-carbamate	100 I.P.
U-930	Piperazine hexahydrate	800 I.P.
63-L	1-Phenylpiperazine hydrochloride	100 I.P.
56-L	1-Ethylpiperazine hydrochloride	100 I.P.
U-910	1,4-Diaminopiperazine hydrochloride	100 I.P.
222-C	1,4-Dimethylpiperazine dihydrochloride	100 I.P.
225-C	1-Carbamyl-4-methylpiperazine	100 I.P.
228-C	1-Methyl-4-thiocarbamylpiperazine hydrochloride	100 I.P.
309-C	Omega-[1-(4-methylpiperazyl)]-propiophenone dihydrochloride	12.5 I.P.
240-C	2,3-Diphenylpiperazine dihydrochloride	100 I.P.
77-L	1,4-Diguanylpiperazine sulfate	100 I.P.
214-C	4-Carbethoxymorpholine	200 Orally
184-L	1-Carbomethoxy-4-methylpiperazine hydrochloride	6.25 I.P.
U-994	1-Carbethoxy-4-nitrosopiperazine hydrochloride	50 Orally
145-L	1-Carbethoxy-4-isobutylpiperazine	100 Orally
224-C	1-Carbethoxy-4-(n-heptyl)-piperazine	200 Orally
193-C	1-Carbethoxy-4-phenylpiperazine	25 I.P.
209-C	1-Carbethoxy-4-benzylpiperazine hydrochloride	200 Orally
60-L	1-Carbethoxy-4-aminopiperazine hydrochloride	100 I.P.
219-C	1-Carbamyl-4-carbethoxypiperazine	100 I.P.
211-C	1-Carbethoxy-4-allylpiperazine	100 I.P.
192-L	4-(β-sulfanylethyl)-1-carbethoxypiperazine	200 Orally
195-L	1-Diethylcarbamyl-4-carbethoxypiperazine	12.5 I.P.
217-C	1,2-Bis-(1′carbethoxy-4′-piperazine) ethane	200 Orally
196-C	1-Ethyl 4[2-(diphenylacetoxy)ethyl]-piperazine	100 I.P.
153-L	1,1′-Carbonyl-bis-(4-methylpiperazine) dihydrochloride	100 I.P.
168-C	1,4-Bis-(α-phenyl-β-benzoylethyl)-piperazine	100 Orally
U-683	Ethyl (1-carbethoxy-4-piperazine)-acetate	100 I.P.
U-682	1-Carbethoxy-4-(2-hydroxyethyl)-piperazine	100 I.P.
162-L	1-Carbomethoxypiperazine	6.25 I.P.
164-L	1-Carbobutoxypiperazine	6.25 I.P.
165-L	1-Carboisobutoxypiperazine	6.25 I.P.
161-L	1,4-Dicarbomethoxypiperazine	6.25 I.P.
163-L	1,4-Dicarbobutoxypiperazine	25 Orally
166-L	1,4-Dicarboisobutoxypiperazine	6.25 I.P.
159-L	1-Diethylcarbamylpiperazine	6.25 I.P.
197-L	1-Diisopropylcarbamylpiperazine	25 I.P.
198-L	1-Di-n-butylcarbamylpiperazine	25 I.P.
199-L	1-Diisoamylcarbamylpiperazine	25 I.P.
176-L	1-Dibutylcarbamyl-4-methylpiperazine hydrochloride	6.25 I.P.
203-L	1-Phenylcarbamyl-4-methylpiperazine	25 I.P.
293-L	1-Benzylcarbamyl-4-methylpiperazine	25 I.P.
158-L	1-Diethylcarbamyl-4-isopropylpiperazine hydrochloride	6.25 I.P.
160-L	1,4-Bis-diethylcarbamylpiperazine	6.25 I.P.

*Treatment administered twice daily for from two to four weeks.

More extensive data concerning the effects of 1-diethylcarbamyl-4-methylpiperazine hydrochloride (84-L) upon microfilariae in vivo and in vitro are presented elsewhere.[4]

Effect of Piperazines on Adult Filariae in Cotton Rats.—In cotton rats treated with several of the piperazines listed in Tables II and III, autopsies performed at various intervals after cessation of treatment revealed no dead

TABLE II. COMPARATIVE MICROFILARICIDAL ACTIVITY AND TOXICITY OF EIGHTEEN PIPERAZINE DERIVATIVES IN COTTON RATS, USING 1-DIETHYLCARBAMYL-4-METHYLPIPERAZINE HYDROCHLORIDE (84-L) AS A STANDARD

COMPOUND	NAME	MINIMUM EFFECTIVE DOSE AGAINST MICROFILARIAE (MG./KG.)	LD_{50} MICE I.P. (MG./KG.)	APPROXIMATE 84-L EQUIVALENT	APPROXIMATE CHEMOTHERAPEUTIC INDEX
163-C	1-Phenylpiperazine hydrochloride	50 I.P.	140	0.06	2.8
162-C	1-Carbethoxypiperazine hydrochloride	25 I.P.	275	0.125	11.0
217-L	1-Methyl-4-(4'-morpholine carbamyl)-piperazine hydrochloride	12.5 I.P.	?	0.25	?
180-C	1-Carbethoxy-4-methylpiperazine hydrochloride	6.25 I.P.	550	0.5	88.0
59-L	1-Carbethoxy-4-ethylpiperazine	50 I.P.	175	0.06	3.5
82-L	1-Carbethoxy-methylpiperazine	100 I.P.	?	0.03	?
U-653	1-Carbethoxy-4-propylpiperazine hydrochloride	25 I.P.	87.5	0.125	3.5
147-L	1-Carbethoxy-4-isopropylpiperazine hydrochloride	25 I.P.	100	0.125	4.0
U-655	1-Carbethoxy-4-butylpiperazine	50 I.P.	125	0.06	2.5
U-801	1-Carbethoxy-4-s. butylpiperazine	100 Orally	175	0.05	1.75*
61-L	1-Guanyl-4-carbethoxy-piperazine sulfate	50 I.P.	285	0.06	5.6
218-C	Bis-(4-carbethoxy-1-piperazine)-methane	25 I.P.	175	0.125	7.0
169-C	1,4-Dicarbethoxypiperazine	100 I.P.	500	0.03	5.0
76-L	1,4-Dicarbethoxy-2-methylpiperazine	200 Orally	375	0.025	1.9*
182-L	1-Ethylcarbamyl-4-methylpiperazine hydrochloride	6.25 I.P.	2,250	0.5	360.0
152-L	1-Dimethylcarbamyl-4-methylpiperazine hydrochloride	6.25 I.P.	310	0.5 to 1.0	48.0
177-L	1-Diisopropylcarbamyl-4-methylpiperazine hydrochloride	6.25 I.P.	160	0.5	25.6
84-L	1-Diethylcarbamyl-4-methylpiperazine hydrochloride	3.13 I.P.	285	1.0	91.0

*Probably not a true value, since the LD_{50} was determined by intraperitoneal injections and the minimum effective dose by oral administration.

adult worms present in the pleural cavity, and there was no macroscopic or microscopic evidence that any damage whatever to the macrofilariae had been effected. In view of the dramatic effects produced against the microfilariae these results were at first disappointing.

One of the first compounds found to produce suggestive activity against the adult worms was 1-carbethoxy-4-isopropylpiperazine hydrochloride (147-L). Dead worms were found in one of the three rats first used to test this compound, and no adult worms were found in another. The third rat revealed all living worms. Attempts to repeat these results in a larger series of rats failed, because this compound was very toxic to the host and prolonged dosage could not be administered.

Thirty rats treated with various doses of 1-carbethoxy-4-methylpiperazine hydrochloride were then brought to autopsy, and, as illustrated in Table V,

TABLE III. EFFECT OF VARIOUS PIPERAZINE DERIVATIVES AGAINST MICROFILARIAE IN COTTON RATS DURING FIRST WEEK OF TREATMENT

COMPOUND	NAME	DOSE (MG./KG.) B.I.D.	MICROFILARIAE PER 100 FIELDS — DAY 1	2	3	4	5	6	7
163-C	1-Phenylpiperazine hydrochloride	25 I.P.	340	284	310	440	144	250	108
		50 I.P.	92	8	0	60	18	24	0
162-C	1-Carbethoxypiperazine hydrochloride	25 I.P.	180	6	4	6	6	8	6
		50 I.P.	652	252	96	18	14	14	12
		100 I.P.	1,360	84	8	10	4	6	6
217-L	1-Methyl-4-(4'-morpholine carbamyl-piperazine hydrochloride	25 I.P.	268			40			2
		50 I.P.	14						1
		100 I.P.	92			6			0
180-C	1-Carbethoxy-4-methylpiperazine hydrochloride	6.25 I.P.	430	190	56	250	52	8	22
		12.5 I.P.	128	72	60	48	22	12	56
		25 I.P.	160	112	28	38	40	6	14
		50 I.P.	384	40	22	12	4	0	4
		100 I.P.	264	26	8	2	6	8	4
		200 I.P.	168	12	2	1	1	4	0
59-L	1-Carbethoxy-4-ethylpiperazine	25 I.P.	1,580	800			430		360
		50 I.P.	1,940	600			188		100
		100 I.P.	136	6			0		1
82-L	1-Carbethoxy-methylpiperazine	25 I.P.	140	144	132	116	248	308	140
		50 I.P.	212	320	124	124	156	136	84
		100 I.P.	76	14	18	14	12	10	6
U-653	1-Carbethoxy-4-propylpiperazine hydrochloride	25 I.P.	64	28	22		32		14
		50 I.P.	248	160	36		18		14
147-L	1-Carbethoxy-4-isopropylpiperazine hydrochloride	25 I.P.	44	3	10		4		1
		50 I.P.	26	6	1		4		8
		100 I.P.	8	0	0		0		0
U-655	1-Carbethoxy-4-butylpiperazine	25 I.P.	284	656	696		208		336
		50 I.P.	160	18	10		6		12
		100 I.P.	116	10	9		10		4
U-801	1-Carbethoxy-4-s. butylpiperazine	50 Orally	388	468	148		568		340
		100 Orally	140	80	26		28		14
		200 Orally	440	44	8		24		0
61-L	1-Guanyl-4-carbethoxypiperazine sulfate	25 I.P.	380	212		436	272	412	304
		50 I.P.	388	68		18	4	24	6
		100 I.P.	104	12		6	8	6	8
218-C	Bis-(4-carbethoxy-1-piperazine)-methane	25 I.P.	272	68	60				22
		50 I.P.	76	32	6				3
169-C	1,4-Dicarbethoxypiperazine	12.5 I.P.	132	196	116	172	40	120	88
		25 I.P.	188	140	140	60	23	48	128
		50 I.P.	440	552	476	676	348	148	188
		100 I.P.	344	88	38	22	56	36	26
		200 I.P.	740	132	30	6	18	6	34
76-L	1,4-Dicarbethoxy-2-methylpiperazine	50 Orally	568	400	248	92	320	400	160
		100 Orally	292	292	412	76	72	268	116
		200 Orally	52	64	38	10	6	2	8
182-L	1-Ethylcarbamyl-4-methylpiperazine hydrochloride	1.5 I.P.	188	332	188	232	176	76	244
		3.13 I.P.	432	1,060	324	472	712	490	612
		6.25 I.P.	36	72	48	28	4	10	6
152-L	1-Dimethylcarbamyl-4-methylpiperazine hydrochloride	25 I.P.	80	20	2	3	6	4	4
		50 I.P.	1,190	2,180	740	870	960	560	310*
		100 I.P.	172	60	8	12	6	4	1
177-L	1-Diisopropylcarbamyl-4-methylpiperazine hydrochloride	1.5 I.P.	152		28	380	10	104	26
		3.13 I.P.	432		104	136	336	244	180
		6.25 I.P.	60		1	20	10	6	2

*No adult worms were found in the pleural cavity at autopsy. Four worms were found in the abdominal cavity.

TABLE III—CONT'D

COMPOUND	NAME	DOSE (MG./KG.) B.I.D.	MICROFILARIAE PER 100 FIELDS DAY 1	2	3	4	5	6	7
84-L	1-Diethylcarbamyl-4-methylpiperazine hydrochloride	1.5 I.P.	480	400	340	270	290	210	184
		3.13 I.P.	1,220	108	40	56	4	8	2
		6.25 I.P.	600	116	44	22	72	40	22
		12.5 I.P.	180	64	8	8	8	1	8
		25 I.P.	396	100	20	8	10	6	1
		50 I.P.	476	76	22	13	12	2	3
		100 I.P.	192	12	6	5	4	10	1
		5 Orally	228	14	0	10	3	2	4
		10 Orally	408	10	6	10	3	2	1
		25 Orally	168	8	0	0	0	0	0
	Nontreated controls	–	1,630	252	590	1560	224	1020	1180
			46	20	28	18	44	40	72
			36	26	56	44	52	58	36
			40	60	88	22	40	24	56
			560	212	208	184	280	244	410
			120	248	348	100	404	628	780
			40	24	24	10	12	42	56

the effects upon the adult worms were extremely variable. Most of the adult worms were alive, however. Similar results were obtained on a smaller series of animals treated with other piperazines effective against microfilariae.

Nine of sixty-five untreated rats which were autopsied showed one or two dead adult worms at autopsy, but in no case were the majority or all of the worms dead. The effects produced by several of the piperazines upon the adult worms in a few rats in each series, therefore, suggested that a wide variety of dosage schedules, with autopsies at different periods after cessation of treatment, might throw some light on the amount of drug and the length of time necessary to kill a larger proportion of the adult worms than had been accomplished in earlier trials. The compound selected for these extensive tests was 1-diethylcarbamyl-4-methylpiperazine hydrochloride, since it produced predictable and rapid reductions in microfilariae in oral or intraperitoneal doses as low as 3 mg. per kilogram, was relatively nontoxic to cotton rats, and was made available in large quantities.

Subsequent data obtained with this compound and reported in a following paper showed that frequent oral dosage for several weeks killed a large proportion of the adult worms. None of the other compounds listed in Tables II and III have been studied so extensively, but it is believed that the best balance between activity and toxicity has been achieved with 1-diethylcarbamyl-4-methylpiperazine hydrochloride. For the reasons described, the activity of the piperazines listed in Tables II and III is based entirely upon their effectiveness against microfilariae. In most cases relatively high toxicity to the host rules out further studies upon their effectiveness against adult worms, and in the few instances where good effects against microfilariae were obtained with relatively nontoxic doses (182-L, 152-L, 180-C) it is felt that further studies would produce little more pertinent information than has been obtained with 84-L.

Effect of Piperazines Upon Dirofilaria Immitis in the Dog.—Several piperazines showing high activity in the cotton rat were tested for effectiveness against

TABLE IV. EFFECT OF 1-CARBETHOXY-4-METHYLPIPERAZINE HYDROCHLORIDE (180-C) AGAINST MICROFILARIAE IN COTTON RATS

RAT	SERIES	DOSE (MG./KG.)	TREATMENT	PER CENT REDUCTION OF MICROFILARIAE	
				7 DAYS	LAST DAY OF TREATMENT
370	RF-32	12½	I.P., b.i.d., 14 days	56.3	59.4
44	RF-9	25	I.P., b.i.d., 14 days	97.5	100.0
38	RF-9	25	I.P., b.i.d., 14 days	70.0	90.0
75	RF-9	25	I.P., b.i.d., 14 days	100.0	100.0
109	RF-9	25	I.P., b.i.d., 14 days	92.3	94.3
70	RF-9	25	I.P., b.i.d., 14 days	25.0	75.0
76	RF-9	25	I.P., b.i.d., 14 days	95.6	94.0
82	RF-9	25	I.P., b.i.d., 14 days	97.8	77.5
133	RF-10	25	I.P., b.i.d., 27 days	100.0	91.7
134	RF-10	25	I.P., b.i.d., 27 days	94.5	77.8
135	RF-10	25	I.P., b.i.d., 27 days	100.0	D.
137	RF-10	25	I.P., b.i.d., 27 days	100.0	14.3
138	RF-10	25	I.P., b.i.d., 27 days	50.0	62.5
372	RF-32	25	I.P., b.i.d., 14 days	91.3	93.8
373	RF-32	25	I.P., b.i.d., 14 days	74.0	88.1
205	RF-16	25	Orally, b.i.d., 28 days	99.6	99.1
210	RF-16	25	Orally, b.i.d., 28 days	99.4	D.
215	RF-16	25	Orally, b.i.d., 28 days	99.4	D.
218	RF-16	25	Orally, b.i.d., 28 days	98.5	99.4
222	RF-16	25	Orally, b.i.d., 28 days	98.2	D.
139	RF-10	50	I.P., b.i.d., 27 days	100.0	100.0
140	RF-10	50	I.P., b.i.d., 27 days	50.0	87.5
141	RF-10	50	I.P., b.i.d., 27 days	83.4	100.0
142	RF-10	50	I.P., b.i.d., 27 days	87.5	100.0
143	RF-10	50	I.P., b.i.d., 27 days	100.0	D.
159	RF-10	50	I.P., b.i.d., 27 days	100.0	100.0
160	RF-10	50	I.P., b.i.d., 27 days	99.4	100.0
374	RF-32	50	I.P., b.i.d., 14 days	100.0	D.
375	RF-32	50	I.P., b.i.d., 14 days	99.0	99.0
207	RF-16	50	Orally, b.i.d., 28 days	100.0	99.3
209	RF-16	50	Orally, b.i.d., 28 days	99.4	D.
216	RF-16	50	Orally, b.i.d., 28 days	95.0	98.8
219	RF-16	50	Orally, b.i.d., 28 days	97.7	98.5
183	RF-15	50	I.P., O.D., 28 days	96.3	100.0
189	RF-15	50	I.P., O.D., 28 days	92.9	100.0
190	RF-15	50	I.P., O.D., 28 days	100.0	96.6
184	RF-15	50	I.P., A.D., 28 days	94.7	D.
187	RF-15	50	I.P., A.D., 28 days	93.4	96.7
197	RF-15	50	I.P., A.D., 28 days	20.0	0
144	RF-10	100	I.P., b.i.d., 14 days	95.0	80.0
145	RF-10	100	I.P., b.i.d., 14 days	99.0	99.0
146	RF-10	100	I.P., b.i.d., 14 days	100.0	D.
148	RF-10	100	I.P., b.i.d., 14 days	100.0	D.
149	RF-10	100	I.P., b.i.d., 14 days	100.0	100.0
376	RF-32	100	I.P., b.i.d., 14 days	97.6	100.0
385	RF-32	100	I.P., b.i.d., 14 days	98.4	100.0
56	RF-11	100	I.P., b.i.d., 29 days	100.0	98.8
131	RF-11	100	I.P., b.i.d., 29 days	100.0	99.6
90	RF-11	100	I.P., b.i.d., 29 days	100.0	100.0
206	RF-16	100	Orally, b.i.d., 28 days	97.3	100.0
208	RF-16	100	Orally, b.i.d., 28 days	98.9	D.
213	RF-16	100	Orally, b.i.d., 28 days	98.8	98.8
221	RF-16	100	Orally, b.i.d., 28 days	98.4	D.
186	RF-15	100	I.P., O.D., 28 days	87.0	78.3
196	RF-15	100	I.P., O.D., 28 days	98.1	94.3
212	RF-15	100	I.P., O.D., 28 days	99.0	100.0
194	RF-15	100	I.P., A.D., 28 days	92.3	69.3
203	RF-15	100	I.P., A.D., 28 days	90.5	54.1
204	RF-15	100	I.P., A.D., 28 days	87.0	98.2
155	RF-19	100	*	D.	D.

TABLE IV—CONT'D

RAT	SERIES	DOSE (MG./KG.)	TREATMENT	PER CENT REDUCTION OF MICROFILARIAE	
				7 DAYS	LAST DAY OF TREATMENT
153	RF-19	100	*	98.3	98.3
157	RF-19	100	*	100.0	100.0
158	RF-19	100	*	D.	D.
98	RF-11	200	I.P., b.i.d., 29 days	D.	D.
124	RF-11	200	I.P., b.i.d., 29 days	100.0	100.0
102	RF-11	200	I.P., b.i.d., 29 days	100.0	100.0
386	RF-32	200	I.P., b.i.d., 14 days	98.0	100.0
387	RF-32	200	I.P., b.i.d., 14 days	100.0	99.3
18	RF-11	200	Orally, b.i.d., 29 days	99.3	100.0
33	RF-11	200	Orally, b.i.d., 29 days	99.2	D.
40	RF-11	200	Orally, b.i.d., 29 days	100.0	D.
248	RF-19	200	*	97.8	97.8
250	RF-19	200	*	100.0	100.0
251	RF-19	200	*	100.0	100.0
252	RF-19	200	*	100.0	100.0
50	RF-11	400	Orally, b.i.d., 29 days	100.0	D.
55	RF-11	400	Orally, b.i.d., 29 days	100.0	100.0
127	RF-11	400	Orally, b.i.d., 29 days	100.0	D.
88	RF-12	Diet 0.5%	15 days	97.5	98.4
92	RF-12	Diet 0.5%	15 days	100.0	98.9
96	RF-12	Diet 0.5%	15 days	91.6	D.

I.P., Intraperitoneal b.i.d., twice daily; O.D., once daily; A.D., once on alternate days; D., death.

*Series RF-19 treated every two hours for forty-eight hours, then twice daily for six more days.

Dirofilaria immitis in the dog. The only two compounds which produced measurable reductions in microfilariae in tolerated doses were 1-carbethoxy-4-methylpiperazine hydrochloride (180-C) and 1-diethylcarbamyl-4-methylpiperazine hydrochloride (84-L). The administration of oral or intraperitoneal doses of compound 180-C which produced reductions of microfilariae (25 to 100 mg. per kilogram twice daily) invariably produced nausea, profuse salivation, and muscular weakness. Compound 84-L was much better tolerated, and subsequent experiments in dogs were confined to this derivative. The results obtained with 84-L in dogs, against both micro- and macrofilariae, are presented in another paper in this series.

DISCUSSION

The effectiveness of several piperazine derivatives (administered orally or intraperitoneally) in rapidly reducing the microfilarial count in filaria-infected cotton rats is unique in the experimental chemotherapy of this infection. If the effectiveness of the piperazines was confined to the microfilariae, it would probably indicate that their potential usefulness for treating filarial diseases in other animals and man would be limited. The fact that several of the piperazines tested showed suggestive action against the adult worms, and that one in particular killed a large proportion of the adult worms when administered frequently in tolerated doses for several weeks, certainly removes this group of compounds from the class of experimental curiosities.

The value of the cotton rat bio-assay method for predicting potential filaricidal activity against related diseases in human beings has not been fully tested.

TABLE V. EFFECT OF 1-CARBETHOXY-4-METHYLPIPERAZINE HYDROCHLORIDE AGAINST ADULT WORMS IN COTTON RATS

RAT	DOSE (MG./KG.)	TREATMENT	MICROFILARIAE PER 100 FIELDS			AUTOPSY (DAYS AFTER LAST TREATMENT)	AUTOPSY RECORD
			DAY 1	DAY 7	DAY OF AUTOPSY		
117	25 I.P.	b.i.d., 7 days	24	4	84	15	Mass of live worms
119	25 I.P.	b.i.d., 7 days	34	0	104	15	Mass of live worms
38	25 I.P.	b.i.d., 14 days	40	6	4	1	Several dead worms; none alive
75	25 I.P.	b.i.d., 14 days	30	0	0	1	Several live and several dead worms
109	25 I.P.	b.i.d., 14 days	52	4	2	1	Several live and several dead worms
70	25 I.P.	b.i.d., 14 days	56	42	8	1	Several live and several dead worms
76	25 I.P.	b.i.d., 14 days	68	3	4	1	Several live and several dead worms
82	25 I.P.	b.i.d., 14 days	132	3	4	1	Mass of live worms
135	25 I.P.	b.i.d., 10 days	18	0	0	1	Several live and several dead worms
143	50 I.P.	b.i.d., 15 days	4	0	0	1	Mass of live worms
146	100 I.P.	b.i.d., 19 days	8	0	0	1	Mass of live worms
141	50 I.P.	b.i.d., 26 days	6	1	0	13	Several live worms
40	200 Orally	b.i.d., 11 days	200	0	4	3	Mass of live worms
90	100 I.P.	b.i.d., 28 days	64	0	424	55	Mass of dead worms; none alive
18	200 Orally	b.i.d., 28 days	132	1	0	8	Several dead worms; none alive
55	400 Orally	b.i.d., 28 days	356	0	40	8	Mass of live worms
215	25 I.P.	b.i.d., 12 days	146	3	2	1	Mass of live worms
209	50 Orally	b.i.d., 13 days	160	1	0	1	Mass of live worms
208	100 Orally	b.i.d., 11 days	348	4	0	1	Mass of live worms
195	1.5 I.P.	b.i.d., 14 days	740	76	428	56	Mass of live worms
198	3.13 I.P.	b.i.d., 14 days	1,840	248	192	56	Mass of live worms
41	6.25 I.P.	b.i.d., 14 days	430	22	52	56	Mass of live worms
104	12.5 I.P.	b.i.d., 14 days	104	16	184	56	Mass of live worms
539	5 Orally	b.i.d., 10 days	1,700	180	528	1	Mass of live worms
527	5 I.P.	b.i.d., 15 days	72	172	2	112	Mass of live worms
522	5 I.P.	b.i.d., 15 days	252	80	380	112	Mass of live worms
532	5 I.P.	b.i.d., 15 days	26	6	84	112	Mass of live worms
530	5 Orally	b.i.d., 15 days	1,080	370	630	112	Mass of live worms
529	5 Orally	b.i.d., 15 days	204	40	24	112	Mass of live worms
540	5 Orally	b.i.d., 15 days	600	112	212	112	Mass of live worms

Culbertson, Rose, and Olive-Gonzalez,[5-7] however, have demonstrated that a correlation exists between the effects produced by Neostibosan against *Litomosoides carinii* in cotton rats and *Wuchereria bancrofti* infections in man.

We believe it to be important, also, that several of the most active piperazines are effective when administered orally. With the exception of malaria and the intestinal helminth diseases, the chemotherapy of parasitic diseases in man is largely confined to the parenteral administration of various drugs, usually compounds containing antimony or arsenic. The value of some of the heavy metals in the treatment of several of the most important parasitic diseases is not disputed, but the advantages of an effective oral treatment for any disease in human beings or domestic animals are obvious, particularly if no toxic effects are produced.

There is a measurable correlation between structure and microfilaricidal activity in the piperazines, as demonstrated in Table II. We cannot be sure that the most active member of the group has been found, and there is room for further investigation in this respect.

In comparison with several antimony derivatives, the piperazines produce a much more rapid effect upon the microfilariae of cotton rats in the peripheral blood.[8, 9] The effect produced against adult worms by piperazines is very slow in comparison with that of antimony salts. An entirely different mode of action must therefore be involved. No studies have been made thus far in attempts to determine how the piperazines produce their effects upon either microfilariae or macrofilariae.

SUMMARY

The effects of various piperazine derivatives against naturally acquired filaria infections in cotton rats and dogs are discussed. Several compounds in this group produce rapid reductions in microfilariae following oral or intraperitoneal dosage, and one in particular produces a lethal effect against adult filariae when administered frequently for a period of several weeks.

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EXPERIMENTAL CHEMOTHERAPY OF FILARIASIS

III. Effect of 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride Against Naturally Acquired Filarial Infections in Cotton Rats and Dogs

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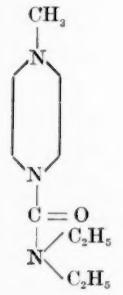
IT HAS been demonstrated in a preceding paper[1] that the intraperitoneal or oral administration of various piperazine derivatives to cotton rats naturally infected with *Litomosoides carinii* causes a rapid and precipitous reduction of microfilariae in the peripheral blood. Of more than one hundred derivatives made available, 1-diethylcarbamyl-4-methylpiperazine hydrochloride (compound 84-L) was selected as the most promising because of its repeated marked effects against microfilariae in both cotton rats and dogs and because of its relatively low toxicity in these animals. Subsequent studies showed that proper dosage regimes killed a large percentage of the adult worms. The structural formula of this compound is given below.



Two hundred twelve cotton rats and twenty-five dogs have been used to test the effectiveness of this compound against both microfilariae and macrofilariae, and the results obtained are reported herein. The methods used for counting microfilariae and for evaluating the effect upon adult worms have been discussed in a preceding paper.[2]

Effect of 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride Against Microfilariae.—After oral or intraperitoneal treatment with this compound in filaria-infected cotton rats, at doses ranging from 3 to 100 mg. per kilogram, the microfilarial count drops precipitously within twenty-four hours (Tables I and II). It then becomes negative or remains at a very low level for as long as treatment is continued.

There may or may not be a recurrence of microfilariae in the peripheral blood upon cessation of treatment (Tables III, V, IX, and X). The extent of

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TABLE I. EFFECT OF 84-L ON THE MICROFILARIAE OF COTTON RATS (INTRAPERITONEAL DOSAGE)

RAT	DOSE (MG./KG., B.I.D.)	MICROFILARIAE PER 100 LOW-POWER FIELDS — DAYS DURING TREATMENT							
		1	2	3	4	5	6	7	14
566	5	92	10	2	2	4	4	6	4
579	5	36	14	1	1	1	0	0	4
571	5	76	8	14	24	22	8	1	1
584	5	48	12	0	0	0	1	8	0
565	10	112	10	6	2	0	0	0	0
583	10	100	52	8	5	22	10	8	6
588	10	232	56	2	6	1	0	0	0
562	10	548	28	8	4	4	4	1	0
567	25	364	30	1	3	1	2	2	1
572	25	692	20	0	0	4	4	0	1
587	25	452	8	1	2	2	0	1	1
548	25	184	36	6	1	1	2	2	0
568	50	22	14	0	0	4	1	0	0
574	50	408	22	2	0	4	0	0	0
542	50	44	26	8	3	6	4	6	1
586	50	52	2	6	0	1	1	0	0
552	100	36	22	8	4	4	4	6	0
576	100	48	6	0	1	2	3	1	0
580	100	52	30	8	12	44	26	6	0
582	100	284	24	4	1	0	1	1	4

TABLE II. EFFECT OF 84-L ON THE MICROFILARIAE OF COTTON RATS (ORAL DOSAGE)

RAT	DOSE (MG./KG., B.I.D.)	MICROFILARIAE PER 100 LOW-POWER FIELDS — DAYS DURING TREATMENT							
		1	2	3	4	5	6	7	14
900	5	48	12	4	5	1	0	1	0
934	5	228	14	0	10	3	2	4	2
897	5	248	26	22	4	8	3	8	8
912	5	152	36	12	20	8	4	2	6
724	5	68	24	6	6	4	3	0	8
926	5	140	10	6	10	0	3	22	4
898	5	56	16	10	4	1	4	0	0
938	5	92	10	4	4	2	0	2	1
791	5	570	12	8	2	2	2	1	3
927	10	44	20	14	6	6	0	2	2
908	10	408	10	6	10	3	2	1	0
909	10	56	14	8	6	1	0	1	1
911	10	400	2	6	6	14	6	4	1
915	10	12	1	4	6	3	0	0	0
709	10	10	10	3	4	6	2	2	1
917	10	1,260	24	4	6	4	4	0	1
919	10	12	2	0	0	1	1	0	0
920	10	156	6	6	0	4	1	0	1
933	10	68	18	3	2	4	2	2	6
895	25	72	3	0	0	0	0	0	0
940	25	168	8	0	0	0	0	0	0
913	25	40	1	2	0	0	1	0	0
925	25	36	1	0	0	0	0	0	0
931	25	160	3	4	1	2	1	0	0
637	25	156	2	1	0	1	0	0	0
924	25	292	12	1	0	0	0	0	0
930	25	72	6	0	0	0	1	0	0
936	25	288	3	0	0	0	2	6	1
921	25	96	3	0	1	0	0	1	0
775	25	224	6	10	6	0	0	1	0
904	25	18	10	0	0	0	0	0	0
905	25	116	1	0	0	0	1	0	1

TABLE III. EFFECT OF 84-L AGAINST FILARIASIS IN COTTON RATS (ORAL TREATMENT EVERY TWO HOURS FOR FORTY-EIGHT HOURS)

RAT	DOSE (MG./KG.)	MICROFILARIAE PER 100 FIELDS						AUTOPSY RECORD
		BEFORE TREATMENT	HOURS AFTER TREATMENT			DAYS AFTER TREATMENT		
			2	12	24	4	11	
1284	25	148	3	0	0	-	-	Killed at 48 hr.; large mass of live worms
1286	25	340	4	0	0	-	-	Killed at 48 hr.; large mass of live worms
1293	25	48	4	1	0	-	-	Killed at 48 hr.; large mass of live worms
1268	25	220	34	1	0	-	-	Killed at 48 hr.; large mass of live worms
1278	25	156	3	0	0	4	16	Killed on 12th day; mass of live worms; several dead worms; small clump of caseous material
1292	25	248	22	2	0	2	6	Killed on 12th day; one large clump of worms on ventral side of heart, most of these dead; small mass of worms under lungs, some of these living, others dead
1253	25	320	22	0	0	0	16	Killed on 12th day; small clump of live worms, some encased in caseous material
1297	25	964	24	0	0	0	18	Killed on 12th day; many dead and degenerating worms; some living worms
1259	25	664	40	3	1	6	30	Killed on 12th day; mass of live worms; several dead worms
1256	10	148	10	0	0	Dead	-	Dead at 36 hr.; no autopsy
1266	10	676	8	8	2	4	12	Killed on 12th day; small mass of worms, some dead
1226	10	16	0	0	0	0	-	Killed on 8th day; small mass of live worms; 3 dead worms; some caseous material present
1246	10	400	30	8	22	-	-	Killed at 32 hr.; mass of live worms; two clumps of dead and disintegrating worms
1255	10	532	8	0	0	0	-	Killed on 8th day; masses of live worms; 1 dead worm
1264	10	248	8	0	0	0	16	Killed on 12th day; large mass of worms; many dead
1265	10	604	22	16	4	9	92	Killed on 12th day; large mass of worms; many living and some dead
1274	10	592	104	44	9	-	-	Dead at 42 hr.; large mass of live worms; several small clumps of dead worms
1267	10	748	52	14	1	2	48	Killed on 12th day; small mass of live worms; large clump of dead worms; caseous material present
1279	5	176	16	2	0	1	46	Mass of live worms
1281	5	192	8	0	0	0	6	Mass of live worms
1271	5	88	48	6	0	6	26	Mass of live worms
1269	5	148	4	0	0	-	-	Dead on 4th day; no autopsy
1258	5	380	204	14	6	4	38	Small mass of live worms
1299	5	324	52	3	0	3	36	Mass of live worms
1275	5	348	18	0	0	-	-	Dead on 4th day; no autopsy
1291	5	276	4	20	0	6	84	Mass of live worms
1300	5	260	80	8	4	-	-	Dead on 4th day; no autopsy

recurrence is related somewhat to the amount and frequency of dosage, although no predictions can be made in this respect. No definite relationship exists between the recurrence of microfilariae and the height of the initial count

(Table V), nor does the height of the initial count affect the rapidity with which the microfilariae disappear (Tables I and II).

The rapidity of the reduction of microfilariae is well illustrated in Table III which gives the results obtained when treatments were administered every two hours. Within two hours after the first oral dose of 25 mg. per kilogram had been administered all animals showed a reduction of over 90 per cent in circulating microfilariae. At the end of twelve hours of treatment (at 5, 10, and 25 mg. per kilogram) many of the rats exhibited no microfilariae in the peripheral blood.

Microfilariae from cotton rats and frogs undergo contortion and spasmodic contractions when placed in solutions of the drug. The microfilariae of *Folyella dolichoptera* from southern frogs[3] demonstrate this effect very well when placed in dilutions of 1:100, 1:1,000, and 1:10,000 of 1-diethylcarbamyl-4-methylpiperazine hydrochloride. These microfilariae are very large embryos with a narrow whiplike anterior and a thicker posterior end. When placed in contact with solutions of the drug, the anterior end of the embryos contracts immediately into a tight coil, and violent, jerky movements occur. They then straighten out and become completely motionless within five to fifteen minutes. The same effect can be observed upon frog microfilariae in vivo during treatment. Similar effects occur with the microfilariae of *Litomosoides carinii*, although to a lesser extent.

These observations, plus the fact that adult filariae removed from cotton rats in early stages of treatment show no demonstrable damage to the embryos in utero, lead us to believe that this compound acts directly upon the microfilariae in the peripheral blood.

TABLE IV. EFFECT OF 84-L ON THE MICROFILARIAE OF DIROFILARIA IMMITIS

DOG	DOSE (MG./KG.)	MICROFILARIAE PER 0.05 C.C. BLOOD — DAYS DURING TREATMENT								
		1	4	7	14	21	28	35	42	49
100	25 I.P., b.i.d.	896	41	30	1	6	3	–	–	–
221	25 Orally, t.i.d.	378	–	19	1	2	1	0	4	2
222	25 Orally, t.i.d.	735	–	29	0	0	2	1	1	2
260	25 Orally, t.i.d.	734	316	39	50	12	27	20	18	–
262	25 Orally, t.i.d.	235	265	–	32	14	4	–	–	–

In dogs infected with *Dirofilaria immitis*, 1-diethylcarbamyl-4-methylpiperazine hydrochloride produces marked reductions in the number of microfilariae during treatment. This effect is most striking in animals with high initial counts (Table IV). The rate of disappearance in dogs is not so rapid as in cotton rats, but within one or two weeks, more than a 90 per cent reduction generally occurs.

Effect of 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride Against Adult Filariae in Cotton Rats.—The effects of treatment with this drug on adult filariae in the cotton rat are not demonstrable so quickly as in the case of the microfilariae. It was apparent from the beginning of this work that demonstrable lethal effects on the adult worms involved a number of variables. The

most important of these have been found to be (1) the amount of drug given, (2) the frequency of dosage, and (3) the number of days elapsing from cessation of treatment to autopsy.

Autopsies performed on untreated rats revealed a small number of dead worms in nine of sixty-five (13.9 per cent). No evidence of massive deaths, adhesions, or large deposits of exudate were found in any of these animals, however, and the number of dead worms formed but a small percentage of the total number present.

TABLE V. COMPARISON OF TREATMENT THREE TIMES DAILY FOR FOURTEEN DAYS AND FOR THIRTY DAYS (84-L., 25 MG./KG.; COTTON RATS)

RAT	MICROFILARIAE PER 100 FIELDS — DAYS 1	7	14	21	28	35	42	51	AUTOPSY RECORD (42ND AND 52ND DAYS)
Treatment Three Times Daily for Fourteen Days									
904	18	0	0	6	1	14	18		Small mass of live worms; 4 dead worms
925	36	0	0	6	20	0	1		15 live worms; small clump of dead and disintegrating worms
913	40	0	0	6	34	56	112		Large mass of live worms; 3 dead worms
895	72	0	0	0	0	0	0		Approximately 30 worms, half of them dead and disintegrating
930	72	0	0	8	8	16	64		Small mass of live worms; 7 dead worms
921	96	1	0	2	0	2	10		Mass of live worms; none dead
905	116	0	1	6	12	42	56		Mass of live worms; none dead
637	156	0	0	1	0	0	0		Mass of live worms; small clump of dead worms
931	160	0	0	4	8	60	10		Large mass of live worms; approximately 20 dead worms
940	168	0	0	14	8	0	0		12 to 15 live worms; 5 to 8 dead worms
775	224	1	0	1	4	0	4		Small mass of live worms; none dead
936	288	6	1	30	38	48	112		Mass of live worms; several dead worms
924	292	0	0	40	140	172	60		Mass of live worms; none dead
Treatment Three Times Daily for Thirty Days									
1079	52	0	0	2	3	12		76	Mass of live worms; several clumps of dead worms
1050	72	0	0	0	0	0		6	Large mass of worms; majority dead
1051	96	1	0	0	0	0		0	Mass of live worms; small clump of dead worms
1089	112	2	2	1	1	0		48	Mass of live worms; small clump of dead worms
1074	124	6	0	0	0	0		0	Extensive adhesions; 1 live worm; 1 dead worm
1057	140	16	3	0	3	37		308	Large mass of live worms; none dead
1069	160	2	0	1	0	8		40	Mass of live worms; none dead
1054	208	0	1	1	3	10		44	Extensive adhesions; clump of dead worms; several living worms
1078	260	3	0	0	0	3		110	Mass of worms; approximately half of them dead
1093	264	2	2	0	0	4		24	Extensive adhesions; several live worms
1070	324	0	0	0	1	1		60	Several live and several dead worms
1094	440	0	0	0	0	4		96	Mass of live worms; one small clump of dead worms

Of 150 rats treated with 1-diethylcarbamyl-4-methylpiperazine hydrochloride, with doses of 10 mg. per kilogram or more, twice or more daily, 72 per cent showed either some or all adults dead at autopsy or no adult worms.

Table VIII presents in summary form the results of autopsies on 212 rats treated with doses of from 3.13 to 100 mg. kilogram twice or more daily for from two to four weeks. Both oral and intraperitoneal treatments have been included since there is no measurable difference in the effect produced by either of these routes of administration. In general, animals treated with less than 10 mg. per kilogram did not exhibit as many dead worms at autopsy as did those treated with 10 mg. or more per kilogram.

The frequency of dosage also influenced the rapidity with which adult worms were killed. In Table III, for example, dead worms were found on the twelfth day in many rats after dosage was administered every two hours for two days. Dosage three times daily has given more consistent results than dosage twice daily.

Dead worms have been found in animals treated for from two to four weeks, and, as illustrated in Table V, there was no real difference in the condition of the adult worms following these treatment periods. A more striking difference occurred in animals subjected to autopsy at different periods after cessation of treatment. This is illustrated in Table IX and by the comparison of Tables V and X. Of the thirty-eight rats for which data are given in Table X, only three (7.9 per cent) showed all living worms at autopsy. These rats were treated orally three times daily for thirty days with 25 mg. per kilogram, and were then held seventy-seven days before autopsy. All these animals showed relatively high initial microfilarial counts, but in many of them no worms were found at autopsy.

More frequently than not, in animals that showed a rapid and sustained reduction in microfilariae during and after treatment, most of the adult worms were dead or none were found at autopsy. This is illustrated in Tables VI and X. Exceptions did occur, however, as demonstrated in Table VII.

Effect of 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride Against Adult Filariae in Dogs.—Twenty-five filaria-infected dogs have been treated thus far with this compound or related derivatives. The results are encouraging in that the doses necessary to produce an effect against the microfilariae, and in some cases against the macrofilariae, do not produce signs of severe toxicity. Moreover, oral treatment is effective.

In Table XI, data are given from six dogs treated with intraperitoneal doses. In two of the dogs (Dogs 94 and 108) no adult worms were found in the heart, pulmonary artery, or lung at autopsy. Both of these animals were killed fifty days after the last treatment, and no microfilariae were found in the blood at the time of autopsy. It will be noted, however, that these animals exhibited relatively low initial embryo counts. It has been shown previously[2] that in many untreated dogs with low initial microfilarial counts the adult worms cannot be found at autopsy in any part of the body. A more certain criterion of cure, therefore, is the presence of dead worms in the terminal branches of the pulmonary artery.

TABLE VI. TREATED ANIMALS IN WHICH DEATH OR ABSENCE OF ADULT WORMS AT AUTOPSY WAS ASSOCIATED WITH CONSISTENTLY LOW EMBRYO COUNTS DURING AND AFTER TREATMENT

RAT	84-L (MG./KG.)	NUMBER OF DAYS TREATED	MICROFILIARIAE PER 100 FIELDS — DAYS										DAY OF AUTOPSY	AUTOPSY RECORD
			1	7	14	21	28	35	42	49	58	107		
481	3 I.P., b.i.d.	16	68	0	2	1	4	4					43	No worms found
488	3 I.P., b.i.d.	16	132	1	1	0	0	1	0				43	2 clumps of dead worms; none living
543	3 I.P., b.i.d.	30	60	0	0	0	0						31	Several dead worms; none living
629	3 I.P., b.i.d.	32	128	3	0	0	0	0	0	0	0		70	No worms found
617	25 I.P., b.i.d.	28	108	0	0	0	1	0	8	1	0		58	2 live worms; 2 dead worms
599	25 I.P., b.i.d.	28	64	0	0	0	0	0	4	1	1		58	4 live worms; 6 clumps of dead worms
593	25 I.P., b.i.d.	30	22	0	0	0	0	2	1				44	3 dead worms; none living
624	25 I.P., b.i.d.	30	88	0	2	1	0	3	4				44	2 dead worms; none living
1050	25 I.P., t.i.d.	30	72	0	0	0	0		6				52	Large clump of dead worms; several living worms
1074	25 I.P., t.i.d.	30	124	0	0	0	0		0				52	Extensive adhesions; 1 live worm; 1 dead
547	25 Orally, b.i.d.	30	26	0	0	0	0						33	Several dead worms; none living
925	25 Orally, t.i.d.	14	36	0	0	6	20	0	1				42	15 live worms; several small clumps of dead worms
895	25 Orally, t.i.d.	14	72	0	0	0	0	0	0				42	25 to 30 worms; half of them dead
940	25 Orally, t.i.d.	14	168	0	0	14	8	0	0				42	12 to 15 live worms; 5 to 8 dead worms
637	25 Orally, t.i.d.	14	156	0	0	1	0	0	0				42	Mass of live worms; several clumps of dead worms
1325	25 Orally, t.i.d.	30	140		0							0	107	2 small clumps of dead worms; none living
1402	25 Orally, t.i.d.	30	330		0							0	107	No worms found
1406	25 Orally, t.i.d.	30	820		0							0	107	No worms found
1409	25 Orally, t.i.d.	30	130		0							0	107	No worms found
1418	25 Orally, t.i.d.	30	124		0							0	107	No worms found
1425	25 Orally, t.i.d.	30	84		0							0	107	No worms found
1511	25 Orally, t.i.d.	30	160		0							0	107	No worms found
1540	25 Orally, t.i.d.	30	145		1							0	107	No worms found
1544	25 Orally, t.i.d.	30	86		0							6	107	No worms found
1553	25 Orally, t.i.d.	30	96		0							0	107	No worms found
1368	25 Orally, t.i.d.	30	56		0							0	107	Two small clumps of dead worms; none living
1403	25 Orally, t.i.d.	30	28		0							0	107	No worms found
1421	25 Orally, t.i.d.	30	210		0							0	107	No worms found
1414	25 Orally, t.i.d.	30	14		6							0	107	No worms found
1453	25 Orally, t.i.d.	30	36		0							3	107	2 live worms; small clump of dead worms
634	50 Orally, b.i.d.	28	56	0	0	0	0	1	4	8	8		58	1 live worm; clump of dead worms
611	50 Orally, b.i.d.	28	44	0	0	0	0	0	0	0	0		58	No worms found
569	50 Orally, b.i.d.	30	16	0	1	0	0						33	No worms found
623	50 Orally, b.i.d.	28	52	0	0	0	0						30	No worms found
552	100 I.P., b.i.d.	30	36	0	0	0	0						33	Several dead worms; none living

552	100 I.P., b.i.d.	30	36	0	0	0	0					30	No worms found
												33	Several dead worms; none living

TABLE VII. TREATED ANIMALS IN WHICH LOW EMBRYO COUNTS AT AUTOPSY DID NOT SIGNIFY DEATH OF THE MAJORITY OF ADULT WORMS

RAT	84-L (MG./KG.)	NUMBER OF DAYS TREATED	MICROFILARIAE PER 100 FIELDS — DAYS									DAY OF AUTOPSY	AUTOPSY RECORD
			1	7	14	21	28	35	42	49	58		
484	3 I.P., b.i.d.	16	224	8	6	7	0	0				36	Several live worms
486	3 I.P., b.i.d.	16	236	2	0	0	1	0				36	Several live worms
487	3 I.P., b.i.d.	16	204	6	4	16	24	64			0	58	Several live worms
480	6¼ Orally, b.i.d.	15	46	10	1	1	2	0		0		49	Mass of live worms
908	10 Orally, t.i.d.	14	408	1	0	6	24	10	8			42	Mass of live worms; 4 dead worms
917	10 Orally, t.i.d.	14	1,260	0	1	0	6	1	0			42	Mass of live worms; small clump of dead worms
548	25 I.P., b.i.d.	30	184	0	0	0	0	0				35	Mass of live worms
921	25 Orally, t.i.d.	14	96	1	0	2	0	2	10			42	All worms alive
775	25 Orally, t.i.d.	14	224	1	0	1	4	0	4			42	All worms alive
1051	25 Orally, t.i.d.	30	96	1	0	0	0	0		0		52	Large mass of live worms; 1 small clump of dead worms
621	50 Orally, b.i.d.	28	608	0	0	0	0	0	0			42	Large mass of live worms
555	50 Orally, b.i.d.	30	100	0	1	0	0	0				35	Mass of live worms

TABLE VIII. EFFECT OF DIFFERENT DOSES OF 84-L ON ADULT FILARIAE IN COTTON RATS, AS COMPARED WITH NONTREATED CONTROLS

DOSE* (MG./KG.)	NUMBER OF RATS	CONDITION OF ADULT WORMS AT AUTOPSY			
		ALL ALIVE (%)	LESS THAN 50 PER CENT DEAD (%)	MORE THAN 50 PER CENT DEAD (%)	ALL DEAD OR NONE FOUND (%)
3.13	34	63.2	14.7	8.8	13.3
5 and 6¼	28	78.7	14.3	7.0	0.0
10 and 12½	27	40.7	48.1	7.4	3.8
25	104	24.0	42.3	15.3	18.4
50 and 100	19	31.5	15.7	26.3	26.5
Total	212	40.5	33.9	13.2	12.4
Nontreated controls	65	86.1	13.9	0.0	0.0

*Including treatments twice or more daily for from two to four weeks. Autopsies were performed at varying periods of time after cessation of treatment.

TABLE IX. EFFECT OF 84-L AGAINST FILARIASIS IN COTTON RATS (AUTOPSIES AT FOUR-, SIX-, AND EIGHT-WEEK PERIODS; TREATMENT FOR FOUR WEEKS)

RAT	DOSE (MG./KG., B.I.D.)	MICROFILARIAE PER 100 FIELDS, WK. 0	1	2	3	4	5	6	7	8	WEEK OF AUTOPSY	AUTOPSY RECORD
623	50 Orally	52	0	0	0	0					4	No worms found
601	25 I.P.	34	1	0	1	0					4	2 live worms; clump of dead and deteriorated worms
600	25 I.P.	64	0	3	1	0					4	Mass of worms, approximately 50% dead
602	25 I.P.	40	2	1	0						3	Several live and several dead worms
606	50 Orally	52	1	0	0	0					4	Several live and several dead worms
607	50 Orally	108	2	0	0	0					4	Several live and several dead worms
615	25 I.P.	22	1	2	1	4					4	Mass of live worms
619	50 Orally	288	1	1	0	0					4	Mass of live worms
593	25 I.P.	22	0	0	0	0	2	1			6	3 dead worms; none living
624	25 I.P.	88	0	2	1	0	3	4			6	2 dead worms; none living
592	25 I.P.	52	0	1	0	0	0	14			6	1 live worm; none dead
620	50 Orally	104	0	1	3	0	4	10			6	6 live worms
621	50 Orally	608	0	0	0	0	0	0			6	Mass of live worms
626	50 Orally	224	0	0	0	1	4	28			6	Mass of live worms
611	50 Orally	44	0	0	0	0	0	0	0	0	8	No worms found
634	50 Orally	56	0	0	0	0	1	4	8	8	8	1 live worm; clump of dead worms
616	25 I.P.	152	1	1	1	0	5	4	3	72	8	2 live worms; mass of dead worms
599	25 I.P.	64	0	0	0	0	0	4	1	1	8	4 live worms; mass of dead worms
618	50 Orally	68	0	0	0	0	6	6	14		8	1 live worm; several dead worms
590	50 Orally	52	1	2	0	0	4	12	28	18	8	Several live worms; several dead worms
617	25 I.P.	108	0	0	0	1	0	8	1	0	8	2 live worms; 2 dead worms
608	50 Orally	100	0	1	1	0	32	22	72	68	8	Several live worms; none dead

TABLE X. EFFECT OF 84-L AGAINST FILARIASIS IN COTTON RATS (ORAL TREATMENT, 25 MG. PER KILOGRAM, THREE TIMES DAILY FOR THIRTY DAYS)

RAT	MICROFILARIAE PER 100 FIELDS, DAYS 1	DAYS 15	DAYS 107	AUTOPSY RECORD (DAY 107, 77 DAYS AFTER LAST TREATMENT)
1406	820	0	0	No worms found
1511	160	0	0	No worms found
1540	145	1	0	No worms found
1544	86	0	6	No worms found
1553	96	0	0	No worms found
1403	28	0	0	No worms found
1421	210	0	0	No worms found
1414	14	0	0	No worms found
1402	330	0	0	No live worms found; probable remnants of dead worms
1409	130	0	0	No live worms found; probable remnants of dead worms
1418	124	0	0	No live worms found; probable remnants of dead worms
1425	84	0	0	No live worms found; some adhesions
1325	140	0	0	No live worms found; two small clumps of dead worms
1368	56	0	0	No live worms found; two small clumps of dead worms
1410	170	1	24	1 live worm; 1 dead worm; probable remnants of other dead worms
1453	36	0	3	2 live worms; small clump of dead worms
1539	200	1	36	5 or 6 live worms; small clump of dead worms
1312	500	1	120	Small mass of live worms; two small clumps of dead worms
1419	340	0	260	Small mass of live worms; 2 or 3 dead worms
1434	370	1	48	Mass of live worms; two small clumps of dead worms
1442	280	0	72	Large mass of live worms; two small clumps of dead worms
1457	400	0	310	Several live worms; several dead and disintegrated worms
1444	320	0	440	Large mass of live worms; one small clump of dead worms
1474	410	0	56	Large mass of live worms; small clump of dead worms
1498	260	0	220	Large mass of live worms; small clump of dead worms
1480	200	2	280	Large mass of live worms; three clumps of dead worms
1513	180	0	14	7 live worms; 2 or 3 dead worms
1525	280	0	400	Mass of live worms; two clumps of dead worms
1536	440	1	96	Small mass of live worms; small clump of dead worms
1424	120	0	72	Small mass of live worms; two small clumps of dead worms
1528	900	0	480	Mass of live worms; two large clumps of dead worms
1530	540	0	112	Mass of live worms; small clump of dead worms
1508	740	0	390	Large mass of live worms; none dead
1512	36	0	0	3 live worms; none dead
1388	440	0	92	2 live worms; none dead
1395	96	1	112	3 live worms; 1 dead worm
1408	560	0	68	2 live worms; 2 dead worms
1478	320	0	48	Small mass of live worms; small clump of dead worms

In three dogs (Table XI, Dogs 101, 95, and 71) no worms were found in the heart at autopsy, but living worms were recovered from various portions of the pulmonary artery, some in the terminal branches. The remaining dog (Table XI, Dog. 100) was killed relatively soon after cessation of treatment because of a severe case of distemper. Live adults were found in the heart of this animal, but others occurred in the pulmonary artery at various levels.

Data from ten dogs treated orally with 84-L are given in Table XII. In two of these dogs (Dogs 191 and 193) no adult worms were found at autopsy. Marked reductions in microfilariae occurred in all animals with high initial counts, and in three dogs (Dogs 222, 260, and 261) many dead worms were found in the terminal lobes of the lungs. Extensive areas of infarction surrounded the plugged vessels. Upon section,* worms in various stages of disintegration were found.

Dog 225 died after two doses of the drug had been administered. This animal had a very high initial microfilarial count, was severely emaciated, and coughed persistently before and during treatment. Within a short time after the first dose was given it was noted that the animal breathed with difficulty and was in considerable distress. It was found dead in the cage shortly after the second dose had been given. At autopsy a large mass of worms was found in the pulmonary artery near the heart, and these completely plugged the vessel. Since this is the only instance in which this condition occurred we are not convinced that it was due to the administration of the drug. It is quite possible, however, that a simultaneous migration of this large mass of worms from the right ventricle to the pulmonary artery, caused by the presence of the drug in the circulatory system, might have caused the death of the animal.

In addition to the effects produced by the drug on the filariae in dogs, it is of interest that most dogs placed under treatment improved markedly in physical appearance. Most of the dogs when received were emaciated, sickly, and unkempt. Good care and adequate food probably accounted for some of their subsequent improvement, but even in dogs with high initial microfilarial counts, a marked gain in weight and disappearance of symptoms which might be attributed to the presence of Dirofilaria took place during and after treatment.

As yet, too few dogs have been used to determine an optimum treatment schedule, but it seems apparent that frequent administration of the drug for several weeks is desirable. Toxicity in dogs with therapeutic doses has thus far been limited to occasional transient nausea.

DISCUSSION

The effect produced against microfilariae in cotton rats and dogs by 1-diethylcarbamyl-4-methylpiperazine hydrochloride is certainly more rapid than that reported for any other known compound. Moreover, to our knowledge, this is the first compound shown to produce this effect in experimental animals when administered by the oral route. No data have been obtained thus far with regard to the rate of absorption and excretion of this com-

*Sections prepared and studied by Dr. Frederick Dessau.

pound, but indirect evidence denotes that it is absorbed rapidly when given either orally or parenterally. The better effects produced by frequent dosage indicate that it either is excreted rapidly or is degraded in the body.

Studies are now in progress regarding the fate of the embryos once they are removed from the peripheral circulation.

The failure of this compound to kill all adult worms in all animals treated may be due in part to individual variations in the absorption of the drug. Furthermore, the state of maturity of adult worms, or the number of worms present in the pleural cavity, may influence the effectiveness of treatment. There is no question concerning the more rapid lethal effect of parenteral doses of several antimony derivatives and cyanine dyes[4-7] against adult worms in cotton rats. We believe it important, however, that for the first time an organic, nonmetallic compound has been found which produces a very marked effect against filariasis in experimental animals when given by mouth.

It should be pointed out that many different dosage regimes have been included in the 212 treated rats for which summary data are given in Table VIII. The best results, as shown in Table X, have been obtained by treating three times daily for thirty days, and then holding the animals for two months before autopsy. In many of the rats treated by this method, no adult worms were found at autopsy, in spite of the fact that high microfilarial counts were present before treatment was initiated. In other rats in this series, varying numbers of dead adult worms were found at autopsy, and in three rats all adult worms were alive. These data, plus the fact that microfilariae sometimes do and sometimes do not recur in the peripheral blood after cessation of treatment demonstrate that a number of unknown variables probably influence the effectiveness of treatment.

The mode of action of this compound against micro- or macrofilariae in the cotton rat is not known. Worms have been found in various stages of decay and disintegration within the pleural cavity after treatment. Worms recovered shortly after death occurred showed no movement when placed in physiologic saline, but no external or internal changes in the appearance of these worms could be noted. Living or dead embryos occurred within the uteri. In other cases, dead worms were found enveloped in caseous, purulent material such as has been described by Culbertson and Rose[4] following treatment with Neostibosan and Neostam. In very late stages of decay the worms occurred in tight clumps, were usually embedded in a firm, yellowish mass, and were fragmented. In the many cases where no worms were found, the chest cavity was clean and no evidence of fragments of dead worms was found. We have assumed that the dead worm tissue is eventually absorbed.

In some cases where large masses of dead worms were found, extensive adhesions of the heart and lung to the walls of the pleural cavity occurred.

The data obtained thus far from filaria-infected dogs treated with 1-diethylcarbamyl-4-methylpiperazine hydrochloride suggest that this drug may prove useful in the treatment of this infection. As in the case of the cotton rats, individual variations occurred in the susceptibility of the worms to treatment. Emphasis has not been placed upon treated cases in which no adult worms

TABLE XI. RESULTS OF TREATMENT OF DIROFILARIA-INFECTED DOGS WITH INTRAPERITONEAL INJECTIONS OF 84-L

DOG	DOSE (MG./KG.)	MICROFILARIAE PER 0.05 C.C. WEEK 0	1	2	3	4	5	6	7	8	9	10	MICROFILARIAE (LAST DAY BEFORE AUTOPSY)	AUTOPSY (DAYS AFTER LAST TREATMENT)	AUTOPSY RECORD
100	25 I.P., b.i.d., for 14 days	896	30	1	6	3							3	16	Killed because of severe distemper; 36 live worms; several in heart, others in pulmonary artery at various levels
101*	50 I.P., b.i.d., for 13 days	354	16	1	13	11	21		62	67			67	41	No worms in heart; 15 live worms in pulmonary artery near heart; 10 live worms in pulmonary vessels in center of lung
95*	50 I.P., b.i.d., for 13 days	130	12	63									63	2	3 live worms in pulmonary artery; 1 live worm in lung, nearly at terminus of blood vessel
94	50 I.P., b.i.d., for 9 days 100 I.P., b.i.d., for 2 days	8	0	2	0	0	3	1			0		0	50	No worms found
108	50 I.P., b.i.d., for 9 days 100 I.P., b.i.d., for 2 days	28	10	18	1	5					0		0	50	No worms found
71	50 I.P., b.i.d., for 13 days	22	5	19	7	11	7	18			38		38	50	No worms in heart; small clump of live worms (3 or 4) in terminal pulmonary vessel

*Treated several weeks previously with 25 mg. per kilogram b.i.d. for fifteen days. Microfilariae count reduced, but relapsed upon cessation of treatment.

DOG	DOSE (MG./KG.)	MICROFILARIAE PER 0.05 C.C. WEEK 0	1	2	3	4	5	6	7	8	9	10	MICROFILARIAE (LAST DAY BEFORE AUTOPSY)	AUTOPSY (DAYS AFTER LAST TREATMENT)	AUTOPSY RECORD
191	25 Orally, t.i.d., for 23 days	1	0	0	3	0	0	0	1	0		0	0	49	No worms found
193	25 Orally, t.i.d., for 23 days	18	2	0	0	1	1	1	2	1		1	1	49	No worms found
194	25 Orally, t.i.d., for 23 days	7	22	5	2	0	2	9	3	2		0	0	49	No worms in heart; one pair of live worms in pulmonary artery
221	25 Orally, t.i.d., for 64 days	378	19	1	2	1	0	4	2	1	0		0	1	No worms in heart; 15 living worms in pulmonary artery and in lung
222	25 Orally, t.i.d., for 64 days	735	29	0	0	2	1	1	2	2	1	1	1	17	No worms in heart; 2 live worms in pulmonary artery; 4 "cysts" in terminal lobe of lung; upon section these were found to contain worms, with various stages of organization of the thrombus; small hemorrhagic infarcts present
225	25 Orally, 2 doses	1,613											-	-	Animal died in afternoon of first treatment day; large mass of live worms in heart and plugged in pulmonary artery; labored breathing before and during treatment
260	25 Orally, t.i.d., for 60 days	734	39	50	12	27	20	18	20	11	2	8	16	30	10 live worms in heart; 10 live worms in pulmonary artery; 2 live worms in lung; lungs show extensive infarction; many worms found in terminal arteries with thrombus formation
262	25 Orally, t.i.d., for 58 days	235	265	32	14	4	26	16	5	11	134	50	172	30	No worms in heart; 9 live worms in pulmonary artery; no macroscopic lesions in lungs
259*	35 Orally, t.i.d., for 27 days	232	17	14	7	3	3						3	4	8 live worms in heart; none found in lung; no lesions found in lung
261*	50 Orally, b.i.d., for 27 days	209	58	124	88	75	43						43	4	3 worms in heart; 1 live worm in pulmonary artery; massive hemorrhage and infarction in lower quadrant of lower right lobe of lung; several hard cystlike structures containing remnants of dead worms; living and dead embryos within these remnants

*Treated several weeks previously with 30 mg. per kilogram of compound 180-C, orally, t.i.d. for forty-seven days. No sharp reduction in microfilariae occurred.

were found at autopsy, since it is well known[2] that adult worms cannot always be found in untreated dogs which show microfilariae in the blood stream, particularly when the microfilariae are few in number. It is believed important, however, that in three dogs with relatively high initial microfilaria counts large number of dead adult worms were found in the terminal blood vessels of the lung following treatment. These dogs showed marked physical improvement during the course of their treatment; all gained weight and the microfilariae had been reduced rapidly in number. A large series of cases has now been put under observation in order to determine an optimum treatment schedule. It is emphasized again that no disturbing toxic symptoms have occurred during treatment, even though the drug was administered in some cases three times daily for two months. The one death which occurred after two doses had been administered was that of a dog with a very heavy worm burden, and this dog had been in obvious distress several days before treatment was started.

The use of piperazines therapeutically in man is not new, although the use to which they have been put previously has largely been abandoned. Hanzlik[8] gives a summary of the literature on the use of piperazine hexahydrate and several similar compounds as urate solvents. Doses as high as 6 Gm. of piperazine hexahydrate have been administered to human subjects without undesirable effects. Other investigators mentioned by Hanzlik, however, report that large doses produce headache, clonic spasms of the extremities, muscular prostration, incoordination, tremor, malaise, and nausea. Piperazine hexahydrate has been reported by several investigators[8] to be rapidly absorbed and excreted by human patients.

SUMMARY

Data are presented which show the effects produced by 1-diethylcarbamyl-4-methylpiperazine hydrochloride on natural infections with filariae in cotton rats and dogs. An immediate and sustained reduction in microfilariae is produced in cotton rats following oral or parenteral administration of the drug. The effect produced against microfilariae in dogs is less rapid but is substantially the same.

Various factors which influence the effects produced on the adult worms in cotton rats are discussed. The maximum number of adult worms are killed when treatment is administered at frequent daily intervals for a period of three weeks or longer, with doses of 10 mg. or more per kilogram.

It is demonstrated that in some Dirofilaria-infected dogs treated with the compound the worms were not present in the heart, but were located in terminal branches of the pulmonary artery; and in three dogs, many worms were dead and surrounded by areas of hemorrhage and infarction.

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STUDIES IN ERYTHROBLASTOSIS FETALIS

I. Activation of the Incomplete Rh Antibody by the Blood Serum of Full-Term and Premature Newborn Infants

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THE failure to demonstrate Rh antibodies in the majority of sera obtained from mothers who have given birth to erythroblastotic children constituted, for a while, one of the major difficulties in accepting the theory of isoimmunization advanced by Levine and co-workers as the cause of erythroblastosis. The discovery of a second type of antibody, variously called "incomplete antibody" by Race[1] and "blocking antibody"* by Wiener,[2] removed one of the many remaining puzzling problems connected with erythroblastosis. Investigations of Diamond, Abelson, and Denton,[3-5] as well as of Wiener,[6] revealed the fact that this new type of antibody agglutinates Rh-positive cells, provided that albumin solution or undiluted serum replaces physiologic saline solution as the diluent.

In spite of quantitative differences, most adult human sera can be used as the diluent for bringing about agglutination of Rh-positive cells by the incomplete antibody. In contrast to this capacity of adult sera, Lubinsky[7] states that umbilical cord serum cannot be used as a diluent. Wiener,[8] in discussing the "bizarre behavior of congenital hemolytic disease, so that some infants do not present signs of hemolysis until several hours or days after birth," tried to find an explanation for the sudden appearance of hemolysis after birth in the following mechanism. He suggests the possibility that the so-called X-protein, described by Pedersen,[9] is involved in the agglutination of Rh-positive cells by the incomplete antibody, and that the late onset of hemolysis is related to the development of this X-protein which does not form until after birth or at the time of birth. Gurevitch, Polishuk, and Hermoni[10] just recently examined umbilical cord serum and the serum of infants up to six months of age and found them devoid of this enhancing property. From this observation they came to the conclusion that there is no evidence for the presumed role of a so-called serum factor or X-protein in the pathogenesis of hemolytic disease of the newborn. Our investigations deal with the characteristics of serum in prenatal and postnatal life with regard to the activation of the incomplete Rh antibody.

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*The term blocking antibody is considered now to be incorrect. Several other terms have been suggested, such as univalent or glutinin. The need for a generally acceptable term is apparent.

EXPERIMENTAL

Several sera containing Rh antibodies of the incomplete variety, obtained from women with erythroblastotic children, as well as a commercially prepared serum* containing potent antibodies of the same type, were at our disposal. In order to determine the activating capacity of the serum of normal newborn infants, the following experiment was performed:

Decreasing dilutions of serum (volume, 0.1 c.c.) of an Rh-negative patient, Mrs. B., who was delivered of an erythroblastotic child, were mixed with 0.1 c.c. of a 3 per cent suspension of Rh-positive blood cells belonging to Group O. The following diluents for both the serum dilutions and the cell suspensions were used: Column a, physiologic saline solution; Columns b, c, and d, normal adult sera belonging to Groups O, A, and B, respectively; Columns e, f, and g, three normal umbilical cord sera. The mixtures were kept in a water bath for one hour at 37° C. and then spun down at a medium speed for two minutes. The results of this experiment are seen in Table I.

TABLE I. AGGLUTINATION OF RH-POSITIVE CELLS OF GROUP O BY INCOMPLETE RH ANTIBODIES, ACTIVATED BY HUMAN ADULT SERUM AND NORMAL CORD SERUM

ANTI-RH SERUM (MRS. B.)	a	b	c	d	e	f	g
	DILUENT						
	SALINE	HUMAN ADULT SERUM			HUMAN UMBILICAL CORD SERUM		
	(0.9 %)	GROUP O	GROUP A	GROUP B	DEG.	SIL.	WEI.
1:5	–	+++	+++	++++	+++	+	+++
1:10	–	++++	++++	++++	+	±	+++
1:20	–	++++	+++	+++	+	–	+++
1:40	–	++++	++	+++	±	–	++
1:80	–	+++	++	++	–	–	+
1:160	–	++	+	++	–	–	+
1:320	–	+	±	+	–	–	–
0	–	–	–	–	–	–	–

–, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; ++++, very strong agglutination.

Table I shows that the serum of Mrs. B. failed to agglutinate Rh-positive cells when saline solution was used as a diluent. If, however, normal adult serum was used, Rh-positive cells were agglutinated by Mrs. B.'s serum up to a dilution of 320. Umbilical cord sera, on the other hand, exhibited a remarkably different picture. Serum Wei. almost equalled adult sera in its ability to produce agglutination. In contrast, serum Sil. was considerably weaker in its activating capacity, and serum DeG. fell somewhere in between the two extremes. It should be mentioned that these three normal umbilical cord sera were selected at random.

Differences in the capacity of cord sera to activate the incomplete Rh antibody of various anti-Rh sera become evident from the following experiment as shown in Table II. For this experiement, serum of Mrs. Mar. (Part I), who had given birth to an erythroblastotic child, was compared with a commercial anti-Rh serum (Part II) which we assume was obtained by arti-

*This serum was purchased from Dade County Laboratories, Miami, Fla. Several different batches were used, the first one being of considerably higher potency than the rest.

ficial immunization of Rh-negative volunteers with Rh-positive cells. The experiment was carried out in the following way:

Decreasing amounts of Rh antisera (volume, 0.1 c.c.) were mixed with 0.1 c.c. of a 3 per cent suspension of Rh-positive Group O cells. As diluents for both the Rh serum dilutions and the cell suspensions the following were used: Column a, physiologic saline solution; Columns b, c, and d, normal adult sera selected at random; Columns e, f, and g, normal cord sera selected at random. The mixtures were kept in a water bath for one hour at 37° C. and then spun down at a medium speed for two minutes. The resulting agglutination is seen in Table II.

TABLE II. AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY TWO ANTI-RH SERA CONTAINING INCOMPLETE RH ANTIBODIES OF LOWER AND HIGHER TITER

	a	b	c	d	e	f	g
	DILUENT						
		ADULT SERUM			UMBILICAL CORD SERUM		
	SALINE (0.9 %)	GROUP O	GROUP A	GROUP B	FO	MA	HU
	Part I						
MRS. MAR.'S ANTI-RH TEST SERUM							
1:5	–	+++	+++	+++	+	–	–
1:10	–	+++	++	++	–	–	–
1:20	–	++	+	+	–	–	–
1:40	–	+	±	+	–	–	–
0	–	–	–	–	–	–	–
	Part II						
COMMERCIAL ANTI-RH TEST SERUM							
1:5	±	++++	++++	++++	+++	+++	+++
1:10	–	++++	+++	++++	+++	++	+++
1:20	–	+++	+++	+++	++	++	++
1:40	–	+++	++	+++	+	+	+
0	–	–	–	–	–	–	–

–, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; ++++, very strong agglutination.

Mrs. Mar.'s serum (Part I) failed to agglutinate Rh-positive cells when normal cord serum was used as a diluent, whereas adult serum brings out the presence of an incomplete Rh antibody of about average titer.

In Part II of this experiment, incomplete Rh antibodies of rather high titer (commercial Rh test serum) were activated by both adult and cord sera, though even in this case adult serum seemed to be somewhat stronger than cord serum.

The differences in results obtained with these two types of sera probably depend upon the titer of Rh antibodies present in the respective anti-Rh sera. However, the mode of production of the Rh antibody may also be a factor. In one case there was a natural production of antibody in a mother with an

erythroblastotic child, whereas in the other case Rh antibodies were presumably produced by artificial immunization.

The question arose as to whether the serum of premature children, because of its immaturity, would be capable of activating the incomplete antibody. Cord sera of premature babies were thus tested in the following manner:

Decreasing amounts of a commercial Rh test serum (volume, 0.1 c.c.) were mixed with 0.1 c.c. of a 3 per cent suspension of Rh-positive cells belonging to Group O. As diluents for both the Rh serum and the cell suspension the following substances were used: Column a, saline solution; Columns b, c, and d, adult sera selected at random; Column e, cord serum from a 6-month-old fetus who died a few minutes after birth; Columns f and g, cord serum from two premature babies of 8 months' gestation; Columns h and i, cord serum from premature babies born one to two weeks before term; Columns j, k, and l, cord sera from full-term babies. The mixtures were kept for one hour at 37° C., shaken slightly, and then spun down at medium speed for two minutes. The resulting agglutinations are shown in Table III.

TABLE III. AGGLUTINATION OF RH-POSITIVE CELLS OF GROUP O BY HIGH TITERED INCOMPLETE RH ANTIBODY ACTIVATED BY HUMAN ADULT SERUM AND THE CORD SERUM OF PREMATURE AND FULL-TERM BABIES

	a	b	c	d	e	f	g	h	i	j	k	l
	DILUENT											
		ADULT SERUM			UMBILICAL CORD SERA							
COMMERCIAL ANTI-RH TEST SERUM	SALINE (0.9 %)	GROUP O	GROUP A	GROUP B	OZ. (6 MO.)	GO. (8 MO.)	PU. (8 MO.)	FR. (2 WK. PREMATURE)	BR. (1 WK. PREMATURE)	KI. (FULL TERM)	CR. (FULL TERM)	RE. (FULL TERM)
1:5	+	++++	++++	++++	±	++	+	+++	+++	+++	++++	+++
1:10	−	++++	++++	++++	−	+	±	+++	++	+++	++++	+++
1:20	−	+++	++	+++	−	−	−	+	±	+++	+++	++
1:40	−	+++	++	+++	−	−	−	±	−	+	++	+
1:80	−	++	±	+	−	−	−	−	−	±	+	−
1:160	−	+	−	±	−	−	−	−	−	−	±	−
0	−	−	−	−	−	−	−	−	−	−	−	−

−, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; ++++, very strong agglutination.

The anti-Rh serum under investigation produced a very weak agglutination of Rh-positive cells when diluted in saline. In normal adult serum, however, strong agglutination of Rh-positive cells occurred, notwithstanding the differences between the individual adult sera used for that purpose. The three cord sera obtained from full-term babies also activated the incomplete Rh antibody in this Rh test serum, the strongest cord serum to the same extent as an average adult serum (see Table II, Part II). In contrast, serum of premature babies was characterized by a definite weakness in activating potency, obviously depending upon the age of the babies. Umbilical cord serum from a 6-month-old fetus acted almost like saline solution, while the cord sera of two 8-month-old fetuses were only slightly better than saline solution. The impression gained from this experiment is that cord sera from babies

born one to two weeks prematurely have a somewhat lower capacity for activating the incomplete Rh antibody than the average cord serum of full-term babies. Yet in many instances even the serum of full-term infants seemed to be completely devoid of this activating capacity when tested against maternal Rh antiserum of low or average titer, as seen in Table II. Obviously a maturation factor plays a definite role in the appearance of the characteristics responsible for the activation of the incomplete Rh antibody, as demonstrated by means of a suitable anti-Rh serum containing a potent antibody of the incomplete variety.

In the preceding experiment the maturation principle was shown to be operating during prenatal life. The following experiment was designed to show the continued maturation of this activating capacity in postnatal life. For this purpose an anti-Rh serum whose incomplete antibody was not activated by normal cord serum was used.

Three different serum specimens were collected from each of two babies: (1) cord serum; (2) serum from venous blood obtained twenty-four hours after birth; and (3) serum from venous blood obtained forty-eight hours after birth. Decreasing amounts of serum (volume, 0.1 c.c.) from Mrs. Matt., who had been delivered of an erythroblastotic child, were mixed with 0.1 c.c. of a 3 per cent cell suspension of Rh-positive cells belonging to Group O. Serum and cell suspensions were diluted as follows: Column a, in saline solution; Column b, in adult serum; Columns c, d, and e, in serum specimens 1, 2, and 3 obtained from full-term baby Vi.; Columns f, g, and h, in serum specimens 1, 2, and 3 obtained from full-term baby Ha. The tubes were kept in a water bath for one hour at 37° C. and then spun down at medium speed for two minutes. Table IV shows the results of this test.

TABLE IV. AGGLUTINATION OF RH-POSITIVE CELLS OF GROUP O BY AN ANTI-RH SERUM CONTAINING INCOMPLETE ANTIBODIES ACTIVATED BY ADULT SERUM AND NOT BY CORD SERUM OF NORMAL FULL-TERM BABIES

	a	b	c	d	e	f	g	h
	DILUENT							
MRS. MATT.'S ANTI-RH SERUM	SALINE (0.9 %)	POOLED HUMAN ADULT SERUM	SERA OF FULL-TERM NEWBORN BABIES					
			VI.			HA.		
			1	2	3	1	2	3
1:40	–	+++	–	++	++	–	–	–
1:80	–	++	–	+	+	–	–	–
1:160	–	++	–	±	±	–	–	–
1:320	–	+	–	–	–	–	–	–
0	–	–	–	–	–	–	–	–

–, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination.

The incomplete Rh antibody present in the serum of Mrs. Matt. is activated clearly by pooled adult serum. The cord serum of the first full-term baby, Vi., is lacking completely in activating power under the experimental conditions chosen. However, serum specimens obtained from the same baby twenty-four hours as well as forty-eight hours after birth reveal the presence of the activating principle, although it is not equal in strength to that of pooled adult serum.

In contrast, the serum of the second baby, Ha., is still negative forty-eight hours after birth. It should be added that in a different experiment not reported in detail in this paper, a third baby's serum failed to activate the incomplete Rh antibody twenty-four hours after birth, but did so forty-eight hours after birth. It is evident from these experiments, therefore, that in some instances the capacity of a baby's serum to activate the incomplete Rh antibody increases in strength soon after birth and is subject to individual variation.

Considering the fact that the average normal cord serum does not activate the incomplete Rh antibody to the same extent as the average adult serum, the theoretical question is raised as to whether the transfusion of erythroblastotic children with adult's blood may be, at times, contraindicated, due to the fact that the infused adult plasma might activate the incomplete antibody present in the baby. The experimental analysis of this problem presents several difficulties. One of them is that the plasma portion of whole blood taken up in citrate or in a.c.d. solutions is diluted by 75 or 120 c.c. of the crystalloid solution per 500 c.c. of whole blood, resulting in a definite dilution of the blood plasma originally present. In a few experiments it was found that citrated plasma, when compared with serum obtained from the same patient, did not activate the incomplete antibody as well as the serum itself and that the addition of as little as 1 part of saline solution to 2 parts of serum reduced the activating power of the serum remarkably.

Samples of blood obtained from erythroblastotic babies at different periods during exchange transfusions were tested to determine their activating capacities. It was anticipated that with progress of the transfusion the activating capacity of the samples would increase due to the increased percentage of adult plasma in the samples withdrawn. The results of this study in three instances are too irregular to permit the drawing of any final conclusions. Further investigations are needed to elucidate this point.

COMMENTS

On the whole, serum obtained from the umbilical cord of full-term infants, when compared with the average normal adult serum, is characterized by a decreased capacity to activate the incomplete Rh antibody. However, considerable variations do occur. Some adult sera are rather potent in this respect, while others are of relatively low potency. There are a few cord sera which are as strong as adult sera of low or average potency, while other cord sera are weaker than the weakest adult sera tested so far. The susceptibility of the red blood cells used also varies, inasmuch as certain cells are clumped by the incomplete antibody more readily than others. As pointed out by other investigators, agglutination of red blood cells by the incomplete Rh antibody rarely reaches the strength of agglutination frequently observed by the interaction of a strong saline Rh antibody with the corresponding red blood cells.

The serum protein, albumin, and globulin levels were determined on several adult and normal cord sera obtained from full-term babies used in these experiments. In general, the average cord sera contained about 1 Gm. per cent less

of total protein and ½ Gm. per cent less of both albumin and globulin than was found in the average adult serum. However, some of the cord sera which poorly activated the incomplete Rh antibody had serum albumin and globulin levels comparable to those of adult sera which activated the incomplete antibody well. Furthermore, there were no striking differences between protein fractions in the cord sera which did and did not activate the incomplete antibody. Studies of serum protein fractions by more complete methods might give further information.

The experiments reported were carried out by warming the tubes in a water bath at 37° C. This temperature, however, is not essential; room temperature and icebox temperature seem to be equally suitable. The length of incubation necessary to secure maximal agglutination also varies, and while in our experiments the blood-serum mixtures were allowed to stand for one hour, we have the impression that one-half hour is sufficient.

The time and speed of centrifugation are of great importance and determine in many instances the degree of agglutination obtained. The advantage of increased agglutination following prolonged centrifugation at higher speed is frequently outbalanced by the necessity for shaking sediments more intensely at the time of reading, resulting in the breaking up of the clumped cells.

There are certain clinical implications which present themselves as a result of observations reported in this paper. The activating power of serum is apparently dependent upon a maturation factor. Serum of a 6-month-old fetus was practically devoid of activating potency, and the serum of a 7- to 8-month-old fetus showed definitely less activating potency than the cord serum of most full-term babies. The maturation factor becomes apparent and can be easily demonstrated if certain high titered anti-Rh sera, whose incomplete antibody can be activated also by the average normal cord serum, are used. The low titered anti-Rh sera which are not activated even by the normal average cord serum are not useful for the demonstration of the maturation principle during prenatal life. It seems reasonable to entertain the possibility that a certain relationship exists between the capacity of the baby's serum to activate the incomplete antibody and the clinical picture of erythroblastosis. The development of this maturation factor might explain the fact that clinical manifestations become evident only during the second part of pregnancy. In some instances babies are born apparently normal and develop clinical manifestations shortly after birth. This might in some way be related to a rapid increase in activating potency after birth, as was found in several instances.

Because of individual variation, it is not possible to predict the actual status of the activating power of the fetus' serum at any given time. However, the assumption that the factor constantly increases in potency during the last half of pregnancy is justified. Undoubtedly, therefore, the babies are exposed both to the mother's Rh antibody passing from the maternal into their own circulation and to the development of an increased activating capacity of their own blood plasma. The level of activating properties of the serum of erythroblastotic babies examined so far was not appreciably higher than that

of the sera of normal infants of the same age. The school of investigators favoring premature delivery as a therapeutic measure in erythroblastosis fetalis might find additional support for their belief in the observations reported. However, careful clinical judgment should be applied in order to avoid adding the trials of prematurity to erythroblastosis. The induction of premature labor three to four weeks before term in cases where there are reasons to suspect an erythroblastotic baby deserves serious consideration by the obstetrician, who must ultimately make the decision after analysis of the whole problem.

The question of treating erythroblastotic babies with transfusions of adult blood deserves critical thought. Even though it is not possible as yet to prove experimentally an increase of activating potency in the baby's blood plasma following the injection of whole blood, the conclusion that adult plasma given to erythroblastotic babies might possibly increase the danger of intravascular agglutination and hemolysis seems inescapable. An exchange transfusion replacing the larger part of the baby's blood cells would therefore seem to have promise of better results than the simple transfusion of adult whole blood. Furthermore, the transfusion of blood minus plasma (washed red cells) deserves consideration, especially in the milder cases. That even relatively small amounts of adult serum mixed with newborn serum increase the activating power of that newborn's blood serum will be described in a subsequent communication.[11]

CONCLUSIONS

1. The cord sera of full-term newborn babies are compared with normal adult sera regarding their relative capacities to activate the incomplete Rh antibody and produce agglutination. The results depend upon the type of anti-Rh serum selected for this purpose. Certain anti-Rh sera containing incomplete antibodies of low or even average titer, originating from patients with erythroblastotic children, were activated by normal adult sera, but only slightly, or not at all, by normal cord sera. In contrast, other anti-Rh sera, such as those supposedly produced by immunization of volunteers and containing incomplete Rh antibodies of fairly high titer, were activated by both normal adult serum and normal cord serum. However, even in the latter instance, the average normal cord serum frequently proved to be of somewhat weaker potency than the average normal adult serum.

2. A considerable difference could be demonstrated between the cord serum of babies born prematurely and those born at term when anti-Rh sera containing incomplete Rh antibodies of high titer were used. The cord serum of a 6-month-old fetus seemed to be lacking completely in activating potency, resembling saline solution in this respect. The cord serum of babies born four to eight weeks prematurely exhibited some degree of activating potency though less than the cord serum of full-term babies. The activating power of serum, therefore, depends upon a maturation principle which is subject to considerable individual variation.

3. The maturation of the activating capacity may continue after birth. In order to demonstrate this principle, anti-Rh sera containing incomplete anti-

bodies which fail to produce agglutination with cord serum of full-term babies should be selected. Using anti-Rh serum of this type, differences in the activating power of normal cord serum and normal adult serum become readily apparent. Serum specimens from two of three normal babies taken twenty-four to forty-eight hours following delivery showed an increase in activating power when compared with their respective cord serum, although they had not reached the strength of normal average adult serum. Further studies of the maturation factor in postnatal life are necessary.

4. Considerable individual differences in the activating potency of various sera, cord as well as adult, and differences in the susceptibility of various individual blood cells to agglutination influence the titer of agglutination obtained. The theoretical implications of the reported observations with respect to the development of the clinical manifestations of erythroblastosis and the treatment of the disease by means of transfusions of whole adult blood are discussed.

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STUDIES IN ERYTHROBLASTOSIS FETALIS

II. Investigations on the Detection of Sensitization of the Red Blood Cells of Newborn Infants With Erythroblastosis Fetalis

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THE laboratory support of the clinical diagnosis of erythroblastosis fetalis is of ever-increasing importance in order to establish the diagnosis with certainty as early as possible. This need has become more important now that exchange transfusions have been introduced as a method of treatment in severe cases during the first hours of life.

The demonstration of Rh antibodies in the mother's serum is the first indication that she may give birth to an erythroblastotic child, and an increase in this antibody titer during the last half of pregnancy strongly suggests the probability of such an event but does not prove its inevitability. Free circulating Rh antibodies are often found in the umbilical cord serum of erythroblastotic babies. Their demonstration is frequently interpreted as a sign of antibody surplus, indicating that the Rh antigens present in the baby's cells have been completely saturated with the corresponding antibodies. Nevertheless, the presence of these abnormal antibodies in the cord serum is good indication that the baby has been sensitized and might possibly constitute sufficient reason for an immediate exchange transfusion. However, many of the patients with erythroblastosis whom we have had occasion to observe recently showed either no free circulating antibodies or only antibodies of low titer in the cord serum in spite of the fact that these babies exhibited clinical signs of severe blood destruction. In the absence of demonstrable Rh antibodies in the cord serum the laboratory evidence of the baby's sensitization rests upon the demonstration of sensitization of his red blood cells.

Several investigators have described different methods of demonstrating sensitization of the babies' blood cells. Carter and Loughey[1] reported that they succeeded in releasing anti-Rh agglutinins from their attachment to the blood cells of erythroblastotic babies by heating the cell suspensions in a water bath at 56° C. To demonstrate this cell sensitization, Coombs, Mourant, and Race[2-4] use an antihuman globulin rabbit serum which induces the agglutination of erythroblastotic babies' cells but not of normal blood cells. Hill and Haberman[5] have further demonstrated the usefulness of the Coombs test, and they call it the "developing test." Diamond and Abelson have recommended a test for the

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detection of the sensitization of babies' blood cells which they call the "reagglutination test."*

In a preceding paper[6] observations are recorded demonstrating that sera of premature babies as well as many cord sera of full-term babies were characterized by their relative weakness to activate the incomplete Rh antibody, which was in striking contrast to the capacity of adult serum to activate this antibody. With this in mind the following experiments were designed to determine whether normal adult serum would produce agglutination of red blood cells sensitized with the Rh antibody.

EXPERIMENTAL

The first four experiments describe typical procedures followed by us for determining sensitization of the blood cells of erythroblastotic babies. A patient, Mrs. Le. (Rh-negative, Group A), with a history of having given birth previously to erythroblastotic children was again delivered of a child exhibiting severe clinical manifestations of this disease. The cord serum of this baby was examined for the presence of free circulating Rh antibodies, the mother's serum taken during delivery being used both for comparison and as a control. The experiment itself was carried out in the following way:

Decreasing amounts of the serum of Mrs. Le. (Columns a and b) as well as cord serum of Baby Le. (Columns c and d) (volume, 0.1 c.c.) were mixed with 0.1 c.c. of a 3 per cent cell suspension of Rh-positive Group O cells. In Columns a and c, physiologic saline solution was used as a diluent for both the serum dilutions and the cell suspensions. In Columns b and d, normal adult serum of Group AB serve as a diluent. The tubes were shaken thoroughly and kept in the water bath at 37° C. for one hour. Then they were centrifuged at medium speed for two minutes. The resulting agglutination is shown in Table I.

TABLE I. AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY MRS. LE.'S AND BABY LE.'S BLOOD SERA

	a	b	c	d
	MRS. LE.		CORD SERUM	
PATIENTS' SERA	DILUENT			
	SALINE	SERUM	SALINE	SERUM
Undil. 0.1 c.c.	++	+++	−	−
1:2	++	+++	−	−
1:4	++	+++	−	−
1:8	+++	+	−	−
1:16	+++	+	−	−
1:32	+++	+	−	−
1:64	+	+	−	−
1:128	±	±	−	−
1:256	−	−	−	−
0	−	−	−	−

−, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination.

*According to a personal communication, this test is carried out as follows: "The baby's cells obtained from cord blood are washed twice with saline. A thick drop of the baby's washed cells is placed on a slide and mixed with a slightly larger drop of whole oxalated blood from a known Rh-positive donor of a compatible blood group. The mixture is rocked for two minutes on a viewing box and at the end of two minutes bound or blocked cells will agglutinate the Rh-positive blood of the donor."

The Rh antibody titer of Mrs. Le.'s serum was 128, irrespective of whether saline solution or normal adult serum was used as a diluent. In contrast, no Rh antibodies were demonstrable in the cord serum under the experimental conditions employed. In view of the baby's severe clinical manifestations of erythroblastosis, however, it had to be assumed that the baby's red blood cells were sensitized.

Suspensions of red blood cells in this and in subsequent experiments were prepared from cord blood by the following method:

Umbilical cord blood was allowed to stand until clotted. The clot was then loosened from the sides of the test tube and centrifuged in order to precipitate the clot of cells and separate the serum. After the larger part of the supernatant serum had been decanted, the blood clot was broken up with wooden applicators and the cells resuspended in the small remaining portion of supernatant serum. In other instances, the blood clot was squeezed through several layers of gauze in order to obtain a suitable cell suspension. If the suspensions of blood cells were not homogenous, they were filtered through a paper filter of a type that allowed the passage of red blood cells. (The paper filter was first moistened with cord serum, if available; if not, with saline solution.) Finally, the cell suspension was spun down thoroughly, the supernatant being removed as completely as possible by means of a dropping pipette. The cell sediment was not submitted to any washing procedures.

In order to demonstrate the sensitization of the blood cells under investigation, the following experiment was carried out:

Packed Baby Le.'s cells, 0.0125 c.c., were mixed in test tubes with 0.3 c.c. of: (1) three different normal adult sera (Columns a, b, and c), (2) two normal cord sera (Columns d and e), and (3) cord serum of Baby Le. (Column f). The mixtures were shaken thoroughly and kept for one hour in a water bath at 37° C. They then were shaken slightly and spun down by centrifugation for two minutes at medium speed. The results of this experiment are seen in Table II.

TABLE II. TEST TUBE AGGLUTINATION OF BABY LE.'S CELLS SUSPENDED IN VARIOUS ADULT SERA AND IN CORD SERA, RESPECTIVELY

a	b	c	d	e	f
SERA TESTED					
NORMAL ADULT SERA			NORMAL CORD SERA		CORD SERUM OF BABY LE.
+	+++	±	−	−	−

−, No agglutination; ±, faint agglutination; +, slight agglutination; +++, strong agglutination.

When Baby Le.'s cells were suspended in three normal adult sera in test tubes, varying degrees of agglutination occurred. However, when cord serum, including that of Baby Le., was used as a diluent, there was no agglutination. Obviously, therefore, Baby Le.'s cells were sensitized, but visible agglutination did not occur unless the baby's cells were suspended in adult sera which are suitable for activating the agglutination of Rh-positive cells sensitized by the

incomplete Rh antibody. The specificity of the agglutination observed becomes evident from the following experiment:

Packed cells, 0.01 c.c., obtained from three different sources (Column a, blood cells of Baby Le.; Column b, normal adult blood cells, Group O, Rh-positive; and Column C, normal adult blood cells, Group A, Rh-positive) were mixed, in the first row, with 0.3 c.c. of normal serum Group A and, in the second row, with 0.3 c.c. of Group A citrated plasma originating from the same individual from whom the normal Group A serum was obtained. The experiment was carried out in two parts, Part I being kept in the water bath for one hour at 37° C. and Part II in the icebox for one hour at 4° C. The tubes were then spun down in the centrifuge at medium speed for two minutes. The resulting agglutinations are shown in Table III.

TABLE III. TEST TUBE AGGLUTINATION OF BABY LE.'S CELLS BY NORMAL ADULT SERUM AND PLASMA, RESPECTIVELY

	PART I (1 HR., 37° C.)			PART II (1 HR., 4° C.)		
	a	b	c	d	e	f
	CELLS					
	BABY LE.	BL.	MO.	BABY LE.	BL.	MO.
Serum Group A 0.3 c.c.	+++	–	–	++	–	–
Plasma Group A 0.3 c.c.	++	–	–	±	–	–

–, No agglutination; ±, faint agglutination; ++, marked agglutination; +++, strong agglutination.

The specificity of the agglutination of Baby Le.'s cells suspended either in normal adult serum or in normal adult citrated plasma becomes apparent. Two cell suspensions from normal individuals similarly prepared failed to show any agglutinations. In this experiment agglutination of the baby's cells appears to be stronger at body temperature than at icebox temperature.

The agglutination of Baby Le.'s cells in normal adult serum could also be shown very clearly on the slide. As a matter of fact, the slide technique which was carried out in the following manner proved to be most valuable for demonstrating sensitization of this baby's cells.

With a wax pencil, two rings about 2 cm. in diameter were made on a clean microscope slide. In the center of one ring two large loopfuls (or their equivalent) of packed washed Baby Le.'s cells were placed. In the second ring the same amount of packed normal adult Rh-positive cells of Group O was placed. One large drop of normal adult serum of Group A* was added to each ring and the mixtures were stirred with wooden applicators. The slide was constantly tilted back and forth by hand, or was placed on a viewing box as recommended by Diamond, and rocked for a few minutes. Frequently five to ten minutes elapsed before agglutination occurred.

An alternative method to the one described consisted of the following: One large drop of adult serum of Group A was placed in each of two rings on a slide. Two wooden applicators were placed in the test tube containing packed

*Since Baby Le. belonged to Group A, serum of Group A or of Group AB was used in all experiments.

Baby Le.'s cells. The sticks were removed and placed into the first drop of serum on the slide. Sometimes this was repeated, using the other ends of the sticks, in order to insure at least a 10 to 20 per cent cell suspension. Normal Rh-positive Group O cells were added to the second drop of serum in the same manner.

The agglutination of the baby's cells never reached the stage of one solid clump. Instead, agglutination was observed at first only with a magnifying glass, and finally, as time elapsed, ended with the production of many clumps of larger size. Oxalated adult plasma also proved very suitable for demonstrating sensitization. Agglutination of Baby Le.'s cells when taken up in a drop of oxalated plasma on a slide occurred faster and appeared to be even stronger than when taken up in a drop of serum. However, normal cells, too, agglutinated occasionally in oxalated plasma. The addition of one drop of normal saline solution to these latter preparations resulted in a dispersion of the agglutination of the normal cells but did not disturb the agglutination of Baby Le.'s cells. Nevertheless, because of its possible nonspecific effect we feel that oxalated plasma should not be used routinely for this purpose and that serum is the diluent of choice.

Since the cord serum of Baby Le. did not produce agglutination of his own sensitized cells, the following experiment was conducted to determine whether mixtures of Baby Le.'s serum and normal adult serum would induce agglutination of Baby Le.'s cells in view of the fact that this would have important clinical implications. Several mixtures of Baby Le.'s cells with various dilutions of normal adult serum in his own serum were prepared:

1 part of normal adult serum of Group A plus 29 parts of cord serum (Baby Le.).
1 part of normal adult serum of Group A plus 9 parts of cord serum (Baby Le.).
3 parts of normal adult serum of Group A plus 7 parts of cord serum (Baby Le.).
1 part of normal adult serum of Group A plus 1 part of cord serum (Baby Le.).
3 parts of normal adult serum of Group A plus 1 part of cord serum (Baby Le.).

Two rings about 2 cm. in diameter were drawn on each of four microscope slides with a wax pencil. One large drop of each of the described five mixtures was placed in five separate rings. One drop of undiluted cord serum (Baby Le.) was placed in the sixth ring and one drop of the same normal adult serum undiluted in the seventh ring. The eighth ring contained one drop of 20 per cent albumin solution. To each of these drops, Baby Le.'s packed cells were added by means of wooden applicators as described previously. The mixtures were stirred and constantly rocked on a viewing box and read after ten minutes. Table IV shows the results of this experiment.

TABLE IV. SLIDE AGGLUTINATION OF BABY LE.'S CELLS BY VARIOUS MIXTURES OF CORD SERUM AND NORMAL ADULT SERUM

a	b	c	d	e	f	g	h
SERA TESTED							
SERUM MIXTURES					CORD SERUM	ADULT SERUM	ALBUMIN 20%
I	II	III	IV	V			
−	+	++	+++	++++	−	++++	−

−, No agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; ++++, very strong agglutination.

Undiluted normal adult serum produced strong agglutination of Baby Le.'s cells, whereas Baby Le.'s own cord serum failed to do so at all. The addition of increasing amounts of adult serum to the cord serum results in mixtures which have an increasing capacity to agglutinate the sensitized cells. It is interesting to note that a mixture consisting of as little as one part of normal adult serum and nine parts of cord serum agglutinated Baby Le.'s cells even though the degree of agglutination was rather weak. Baby Le.'s cells were not agglutinated in 20 per cent albumin solution under these experimental conditions, an observation which we made repeatedly. Obviously, albumin solution was not a suitable medium for bringing out the agglutination of Baby Le.'s sensitized cells in the slide test.

The experimental results obtained with the serum and cells of Baby Le. may be considered to be typical of erythroblastotic babies whose cord sera do not contain any demonstrable free antibodies. The following three experiments deal with a case in which free Rh antibodies were found in the cord serum.

Mrs. Bar. (Group A, Rh negative), whose serum during pregnancy showed a rather high titer of Rh antibodies of the incomplete variety, was delivered at term of a baby exhibiting severe clinical manifestations of erythroblastosis. The baby belonged to Group A and was Rh positive. An exchange transfusion was successfully performed using Rh-negative, Group A blood. A sample of blood was obtained after each infusion of about 60 c.c. of the donor's blood. Nine blood specimens were collected in Wassermann tubes at regular intervals during the exchange transfusion, the first one being taken before the transfusion started, the last one at the end of the transfusion. The first four blood specimens failed to clot, whereas the remaining ones did, possibly due to the heparin used. The baby's serum (or plasma) collected at the beginning of the transfusion was examined for Rh antibodies in the manner described in the first experiment (Table I). An Rh antibody of the incomplete variety was found in a titer of 16. No saline Rh agglutinin was present. (The mother's incomplete Rh antibody titer was quite high at the time of delivery, above 250.)

In the experiments to be described, the nine blood specimens obtained from Baby Bar. during the exchange transfusion were spun down and the supernatant sera were removed from the sediments. Inasmuch as the sediments of Sera 5 through 9 consisted of blood clots, cell suspensions were prepared as described in the case of Baby Le. The cell sediments of Sera 1 through 4 were not treated further and were used as such. None of the cell suspensions was washed.

In order to determine if Baby Bar.'s red blood cells were sensitized by Rh antibodies the following experiment was carried out: With a wax pencil, ten rings were drawn on five slides. In the first nine rings one drop each of the nine serum specimens obtained during the exchange transfusion was placed. One drop of normal adult serum of Group A was put into the tenth ring. Baby Bar.'s cells obtained from the first blood specimen, drawn before the blood transfusion was begun, were then added to each drop of serum by means of wooden applicators. The concentration of blood cells finally obtained

was estimated to be about 20 per cent. The mixtures within each ring were stirred thoroughly and then placed upon the viewing box and constantly rocked. After ten minutes the results were noted and are recorded in Table V.

TABLE V. SLIDE AGGLUTINATION OF BABY BAR.'S SENSITIZED CELLS BY NORMAL ADULT SERUM AND BY VARIOUS SERUM SPECIMENS TAKEN DURING AN EXCHANGE TRANSFUSION

Serum 1	++
Serum 2	–
Serum 3	–
Serum 4	–
Serum 5	–
Serum 6	–
Serum 7	–
Serum 8	–
Serum 9	+
Adult serum	+++

–, No agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination.

The serum of Baby Bar. (Serum 1), when added to its own cells, produced agglutination on the slide. Surprisingly enough, however, Sera 2 to 8, taken during the exchange transfusion, failed to do so. The last serum, 9, again gave a weak agglutination. Normal adult serum of Group A, when mixed with Baby Bar.'s cells, resulted in a strong agglutination. At the present time we are unable to explain why the serum specimens obtained during the exchange transfusion failed to produce any visible agglutination of Baby Bar.'s cells.

It is the obvious goal of an exchange transfusion to replace the baby's blood with normal blood free of Rh antibodies. For that purpose Rh-negative blood is used by most investigators. During the procedure the donor's Rh-negative blood cells are constantly mixed in ever-increasing amounts with the baby's Rh-positive blood cells. The presence or absence of Rh-positive blood cells in the blood specimens taken during exchange transfusions may be considered a rough indicator of the success and extent of the operation. To this end the following experiment was carried out on the series of blood specimens obtained from Baby Bar. during the exchange transfusion.

From the blood sediments of the nine specimens obtained from Baby Bar. during the exchange transfusion 2 per cent blood cell suspensions were prepared in physiologic saline solution. As controls, 2 per cent suspensions of normal Rh-negative and of normal Rh-positive cells were used. In the first row of tubes (Column a) 0.05 c.c. of each of the 2 per cent cell suspensions was mixed with 0.05 c.c. of anti-Rh test serum 1 (anti-D, 85 per cent); in the second row (Column b), with anti-Rh test serum 2 (anti-D, 85 per cent), and in the third row (Column c), with 0.05 c.c. of physiologic saline solution. After being shaken thoroughly, the tubes were kept for one hour at room temperature and then centrifuged at medium speed for two minutes. The resulting macroscopic agglutination is shown in Table VI.

The two Rh test sera secured from different sources strongly agglutinated the Rh-positive cells which were used as controls. The first test serum agglutinated Baby Bar.'s cells taken from the first and second specimens, while cells

TABLE VI. TEST TUBE AGGLUTINATION OF VARIOUS BLOOD CELL SUSPENSIONS TAKEN FROM BABY BAR. DURING EXCHANGE TRANSFUSION BY TWO DIFFERENT ANTI-RH TEST SERA

	a ANTI-RH SERUM 1	b ANTI-RH SERUM 2	c SALINE SOLUTION
Blood cell suspension 1*	++	+++	–
Blood cell suspension 2	+	+++	–
Blood cell suspension 3	–	+++	–
Blood cell suspension 4	–	++	–
Blood cell suspension 5	–	++	–
Blood cell suspension 6	–	+	–
Blood cell suspension 7	–	–	–
Blood cell suspension 8	–	–	–
Blood cell suspension 9	–	–	–
Normal Rh-positive cells	+++	+++	–
Normal Rh-negative cells	–	–	–

–, No agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination.

*Sample 1 was obtained before any blood was infused; 40 c.c. of blood were withdrawn from the baby and replaced with about 40 c.c. of donor's blood between each subsequent sample.

from the third specimen were negative with this serum. In contrast, the second anti-Rh test serum strongly agglutinated Baby Bar.'s cells up to and including the sixth specimen; blood cells from the seventh specimen failed to show agglutination. Obviously the strength and quality of the Rh test serum used are of great importance in the detection of the baby's Rh-positive cells in blood mixtures resulting from exchange transfusions. The agglutination of the baby's Rh-positive cells by anti-Rh serum seems to be a practical way of testing for their presence in mixtures consisting of both the baby's blood cells and the donor's blood cells as obtained during exchange transfusion. However, the interpretation of this latter observation is not entirely clear. First, the agglutination of the baby's sensitized cells may not have depended solely on the presence of antibodies in the anti-Rh test serum. It has already been pointed out that normal adult serum will induce agglutination of sensitized red blood cells. Second, Baby Bar.'s umbilical cord serum contained a free circulating antibody, suggesting complete sensitization of Baby Bar.'s Rh-positive cells. The question thus arose as to why Baby Bar.'s cells were still subject to agglutination by anti-Rh sera. In order to elucidate these two points, the following experiment was performed.

Twenty circles (about 2 cm. in diameter) were drawn with a wax pencil on several slides. One drop of commercial anti-Rh serum (recommended for the slide technique) was placed in each of ten rings, one drop of normal pooled adult serum of Group A being placed in a second group of ten rings. To the first nine rings of each group packed cells of Baby Bar. prepared from the nine different specimens obtained during the exchange transfusion were added by means of wooden applicators. The tenth ring of each series was used as a control for which normal Rh-positive cells of Group A were used. The slides were placed on the viewing box, rocked, and the resulting agglutination observed after about ten minutes. Table VII shows the findings of this experiment.

The commercial anti-Rh serum agglutinated Baby Bar.'s cells up to the fifth specimen, while cells taken from the sixth specimen failed to show any

TABLE VII. SLIDE AGGLUTINATION OF VARIOUS BLOOD CELL SUSPENSIONS TAKEN FROM BABY BAR. DURING EXCHANGE TRANSFUSION BY ANTI-RH SERUM AND BY NORMAL ADULT SERUM

	a ANTI-RH SERUM	b NORMAL ADULT SERUM
Blood cell suspension 1	+++	+++
Blood cell suspension 2	++	+++
Blood cell suspension 3	++	++
Blood cell suspension 4	+	+
Blood cell suspension 5	±	±
Blood cell suspension 6	−	−
Blood cell suspension 7	−	−
Blood cell suspension 8	−	−
Blood cell suspension 9	−	−
Normal Rh-positive cells	++++	−

−, No agglutination; ±, faint agglutination; +, slight agglutination; ++, marked agglutination; +++, strong agglutination; ++++, very strong agglutination.

agglutination. However, normal adult serum also allowed the recognition of the baby's sensitized cells up to the fifth specimen, thus resembling very closely the results obtained with the anti-Rh serum. The fact that the serum containing anti-Rh antibodies was no more effective in agglutinating this particular baby's cells than was the serum without antibodies would lead one to conclude that in addition to Rh antibodies the serum itself is an important factor. Until further evidence is obtained we do not recommend that this test be used as a guide as to the amount of blood that should be given in an exchange transfusion.

DISCUSSION

The findings reported in this paper have been essentially confirmed by the last six cases of erythroblastosis which we have studied. All six infants exhibited severe clinical manifestations of the disease. Two died shortly after birth before treatment could be administered, while the other four were treated by means of exchange transfusions and lived.* In all six cases the mothers were Rh negative and the babies were Rh positive. Further studies are necessary to determine the sensitivity of the described methods and the applicability of this technique to the detection of mild forms of erythroblastosis.

Normal adult serum, active as well as inactive, was found to be the most suitable diluent for bringing about the agglutination of sensitized cells obtained from babies with erythroblastosis fetalis, especially when the slide test was used. Why albumin solutions, 20 per cent as well as 30 per cent, were not suitable for this purpose is not quite clear. However, these latter solutions can be used successfully in the test tube. Oxalated plasma also proved to be a good medium, its only disadvantage being that it frequently produced a nonspecific type of pseudoagglutination. Nonspecific reactions with the use of normal adult serum have not been observed while examining the cases under consideration. However, in the study of a larger series of normal cord bloods, pseudoagglutination (rouleaux formation) was occasionally encountered, thus making it necessary to observe the slides microscopically. Therefore, from a diagnostic standpoint, the agglutination obtained must be interpreted with great

*We are very much indebted to Dr. Douglas P. Arnold who performed the four exchange transfusions and furnished us with the material for these studies.

care. The presence of even small amounts of saline solution prevented or reduced the agglutination of the baby's sensitized cells. For this reason, packed unwashed cells were used with undiluted adult serum. Wiener[7] reported the agglutination of red blood cells of erythroblastotic babies when these cells were suspended in their own cord sera. In our experience, cord sera are considerably inferior to normal adult sera in this capacity. One case exhibiting a somewhat different feature from the cases described herein seems to be worthy of mention in this connection. In this instance the preparation of a cell suspension from the baby's cord blood clot was more difficult than usual because of a marked degree of hemolysis. The cells were found to be Rh positive and were considered to belong to Group A_2B. The B property of the baby's cells was strongly and definitely agglutinated by an anti-B isoagglutinin; the presence of the A property, however, was somewhat questionable in spite of the fact that both test sera, produced by immunizing donors with blood group-specific substances, were very potent.[8] When normal serum of Group AB was added to the baby's blood cells, the same weak type of agglutination occurred as was obtained with test serum of Group B, indicating that the agglutination of these cells was not due to the interaction between an A antibody and an A factor, but to the fact that the baby's cells were sensitized with an Rh antibody, thus exhibiting agglutination when suspended in normal adult serum. This child's mother belonged to Group O, and one could have construed very easily an exemption to the Bernstein law of inheritance of the blood groups.* The baby's cells, even when suspended in saline solution and examined under the microscope, already showed in each field occasional clumps consisting of five to ten cells. When the baby's packed cells were added to normal cord serum on a slide, macroscopically visible agglutination occurred. The baby's own cord serum was not available.

As a result of the difficulty experienced in the determination of this baby's blood group, one wonders if similar difficulties might be encountered in the determination of the Rh type of erythroblastotic babies. In many instances the baby's Rh type is considered to be positive because agglutination of the cells occurs following the addition of an anti-Rh serum. The question arises as to whether agglutination of red blood cells from erythroblastotic babies by an anti-Rh serum is actually caused by the Rh antibody proper, or whether it is merely due to the addition of normal adult serum. Surprisingly enough, agglutination takes place even in instances in which the baby's cord serum contains free circulating Rh antibodies which, one might assume, had combined with all the available Rh antigens present in the baby's cells. The interpretation of agglutination tests with the blood cells of erythroblastotic babies, therefore, needs careful observation from the standpoint of specificity.

Adult serum of any group can be used to bring about agglutination of the baby's sensitized cells by the slide technique, provided that the blood group

*In order to avoid this statement being misused legally to question the validity of Bernstein's rule, it should be added that this baby, if it had survived, would soon have replaced its sensitized cells with normal cells and would have shown its true blood group a few weeks after delivery.

corresponds to that of the cells. Since there are considerable individual variations in the suitability of individual adult sera, pools can be prepared from adult sera of the same blood group. If the baby belongs to Group O, no attention need be paid to the blood group of the adult serum to be used. In cases where the baby's blood group is unknown or is questionable, serum of Group AB is the safest one to employ.

From the standpoint of treatment, the observations reported in Table IV may be significant. This experiment showed that the addition of as little as 1 part of adult serum to 9 parts of the baby's cord serum induced macroscopically visible agglutination of the baby's cells on the slide. Thus the transfusion of 30 to 50 c.c. of adult whole blood into a newborn baby might be sufficient to enhance the in vivo agglutination of the baby's sensitized cells, resulting in further damage to them. Experimental evidence supporting this theoretical possibility, however, has not yet been found. What happens to the activating properties of adult plasma following transfusion into an erythroblastotic baby still remains unknown.

CONCLUSION

A simple technique for the demonstration of sensitization of the red blood cells in an erythroblastotic baby is described. Agglutination occurs when the packed blood cells of an erythroblastotic baby are suspended in normal adult serum. This test can be carried out either in test tubes or on microscopic slides, the use of the latter proving more sensitive in our hands. The results obtained from this test indicate that the baby's cells are sensitized by the incomplete type of Rh antibody which does not cause agglutination in saline solution, and only slight agglutination if any, in cord serum. Blood specimens taken from erythroblastotic babies during exchange transfusions were examined for the continued presence of sensitized cells. Some practical aspects concerning the validity and specificity of Rh type and blood group determinations of erythroblastotic babies, as well as the treatment of these babies by blood transfusion, are discussed.

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THE URINARY EXCRETION OF PTEROYLGLUTAMIC ACID AND CERTAIN RELATED COMPOUNDS

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IT BECAME evident in the early stages of the investigations of *Lactobacillus casei* factor (folic acid) that only small amounts were excreted in the urine of normal human subjects.[1, 2] The amount excreted daily was estimated to be less than 1 per cent of the probable dietary intake.[2]

These observations indicated that dietary pteroylglutamic acid was probably broken down before excretion. Later, pure pteroylglutamic acid (PGA) became available for administration as a supplement. It then appeared that the amount of pteroylglutamic acid excreted in the urine increased sharply as the dosage was raised, indicating that the capacity of the body to break down pteroylglutamic acid was limited. A corollary of these observations was that pteroic acid did not appear to be excreted as a breakdown product of dietary pteroylglutamic acid because, on a normal unsupplemented diet, the potency of the urine was low when measured either by *Streptococcus faecalis* R[3] or *L. casei*.[2]

In a study carried out with young men on a normal diet, it was found that the average daily urinary folic acid excretion was about 4 μg as measured by assay with *Str. faecalis* R.[3] Supplementation with a very small amount (90μg) of pteroylglutamic acid daily did not increase the urinary excretion.[4]

The daily urinary excretion of pteroylglutamic acid was found to be 2 to 4 μg, rarely exceeding 5 μg, on ordinary diets.[5] Oral or parenteral administration of pteroylglutamic acid led within twenty-four hours to an excretion of 15 to 75 per cent of the amount administered, depending on the size of the dose; one individual excreted 16 per cent of an intramuscular dose of 1 mg., while with a dosage of about 10 mg. daily, either orally or parenterally, the excretion usually ranged between 35 and 50 per cent. When pteroyltriglutamic acid was administered to a patient with sprue, assay of the urine with *L. casei* and *Str. faecalis* R indicated that pteroylglutamic acid was liberated from pteroyltriglutamic acid and excreted in considerable quantities.

Doses of 5 to 16 mg. of pteroylglutamic acid were fed to nine normal subjects and nine hospital patients.[6] The normal subjects before receiving pteroylglutamic acid had an average daily urinary excretion of between 2 and 3 μg of pteroylglutamic acid. They excreted an average of 28.5 per cent of the administered dose. Most of the excretion took place between the second and eighth hour after dosage. The hospital patients excreted much lower percentages of the administered dose.

Studies with six normal subjects[7] showed that only about 3 μg of pteroylglutamic acid, as measured by assay with *L. casei*, were present in the twenty-

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four hour urine prior to administration of pteroylglutamic acid. When 4 mg. of the compound were given, about 32 per cent of the dose was excreted in the urine in twenty-four hours.

EXPERIMENTAL

Normal adult men were used as subjects. Since it was found that on ordinary diets the pteroylglutamic acid (PGA) content of the urine was very low (between 5 and 10 millimicrograms per milliliter) no attempt was made to control the diet and, except when very small test doses were used, it was unnecessary to apply a correction for the basal value of the urine.

Samples were collected in brown bottles and preserved under toluene in the refrigerator. Folic acid was assayed against a pteroylglutamic acid standard with *Str. faecalis* R,[8] using purine, pyrimidine, amino acid, and vitamin supplements as described by Teply and Elvehjem.[9]

In the first series, the urinary excretion of pteroylglutamic acid was studied following the administration of various dosage levels of a solution of the sodium salt. Two experiments were included in which the excretion of the free acid suspended in water or oil was measured. The results, shown in Table I,

TABLE I. EFFECT OF DOSAGE AND MODE OF ADMINISTRATION ON URINARY EXCRETION OF PTEROYLGLUTAMIC ACID

FORM ADMINISTERED	AMOUNT PGA TAKEN (MG.)	COLLECTION PERIOD OF URINE (HR.)	ROUTE OF ADMINISTRATION	PER CENT OF DOSE EXCRETED IN URINE	AVERAGE
Solution of Na salt	0.1	6	Oral	0, 0, 0.9 0.6	0.4
Solution of Na salt	0.5	6	Oral	0, 12, 25, 19, 3, 14	12
Solution of Na salt	1.0	6	Oral	10, 25, 26, 26	22
Solution of Na salt	2.0	6	Oral	32, 36, 38, 51, 56	43
Solution of Na salt	5.0	6	Oral	45, 49, 49, 44, 48, 52	
				48, 46, 57, 56	49
Solution of Na salt	15.0	24	Oral	81, 92, 57	77
Solution of Na salt	5.0	6	Intravenous	51, 54, 72, 64	60
Suspension in water	5.0	6	Oral	35, 31, 39, 32, 35	34
Suspension in oil*	5.0	6	Oral	15, 15, 17, 24, 28	20

*Mazola oil.

indicated that the proportion which was excreted increased as the dose was raised. The greater part of the excretion took place in the first six hours. A small part of the administered dose was excreted in the next eighteen hours. This is illustrated in the data in Table II. An average of 53 per cent of a

TABLE II. TIME RELATIONS IN THE URINARY EXCRETION OF FOLIC ACID FOLLOWING THE ORAL ADMINISTRATION OF 5 MG. OF SODIUM PTEROYLGLUTAMATE

SUBJECT	PER CENT OF DOSE EXCRETED		(% OF 24-HOUR EXCRETION)
	0 – 6 HR.	6 – 24 HR.	EXCRETION IN FIRST 6 HOURS
1	48	2	96
2	48	5	91
3	52	6	90
4	41	4	91
2	49	9	84
5	47	8	85

dose of 5 mg. was excreted in twenty-four hours, and an average of 90 per cent of this excretion took place in the first six hours. After twenty-four hours the urinary folic acid content returned to the basal level.

In the next series, the excretion of folic acid in the urine was studied following the administration of pteroyltriglutamic acid (pteroyl-γ-glutamyl-γ-glutamyl glutamic acid).[10] This compound has about 3 per cent of the activity of pteroylglutamic acid in the *Str. faecalis* R assay. Urinary excretion values in excess of the total microbiologic activity of the administered dose were presumed to indicate that pteroyltriglutamic acid had been excreted as pteroylglutamic acid. The results, which are given in Table III, led to the conclusion that pteroyltriglutamic acid was readily hydrolyzed in the body to pteroylglutamic acid because the urinary excretion values following the administration of pteroyltriglutamic acid were very similar to those observed with pteroylglutamic acid.

TABLE III. AMOUNTS OF FOLIC ACID ACTIVITY FOUND PRESENT IN THE URINE FOLLOWING ADMINISTRATION OF 7.9 MG. OF PTEROYLTRIGLUTAMIC ACID (EQUIVALENT TO 5 MG. OF PTEROYLGLUTAMIC ACID) WHEN ADMINISTERED AS SOLUTION OF SODIUM SALT

SUBJECT	ROUTE OF ADMINISTRATION	PER CENT OF DOSE EXCRETED (MEASURED AS PGA)		EXCRETION IN FIRST 6 HOURS (% OF 24-HOUR EXCRETION)
		0 – 6 HR.	6 – 24 HR.	
4	Oral	37	9	80
5	Oral	51	11	83
6	Oral	38	5	91
7	Oral	62	12	84
4	Intravenous	58	11	84
4	Intravenous	51	10	84
2	Intravenous	45	7	85

There was some indication of a lag in excretion as evidenced by the proportion of the biologic activity excreted in the 6- to 24-hour period being greater with pteroyltriglutamic acid than with pteroylglutamic acid. An average of 15 per cent of the twenty-four hour excretion was found in the urine collected from the sixth to the twenty-fourth hour following dosage with pteroyltriglutamic acid as compared with 10 per cent following dosage with pteroylglutamic acid. The excretion rate, measured as pteroylglutamic acid, appeared to be the same following dosage by either the oral or intravenous route, which could lead to the assumption that the breakdown of pteroyltriglutamic acid to pteroylglutamic acid in the body could take place with equal rapidity whether or not the triglutamic compound passed into the body through the gastrointestinal tract.

Experiments were also made with pteroyldiglutamic acid (pteroyl-α-glutamyl glutamic acid).[10] This compound has practically no activity in stimulating the growth of either *L. casei* or *Str. feacalis* R, so that any substantial increase of microbiologic activity in the urine could be attributed to pteroylglutamic acid formed by breakdown of pteroyldiglutamic acid in the body. The results are in Table IV. The data indicate that the body possesses the ability to liberate pteroylglutamic acid from pteroyldiglutamic acid. Even more than in the case of pteroyltriglutamic acid, there was a lag in the rate of excretion so that a comparatively large but variable proportion of the administered dose, measured

TABLE IV. EXCRETION OF FOLIC ACID IN THE URINE FOLLOWING THE ADMINISTRATION OF 6.5 MG. OF PTEROYLDIGLUTAMIC ACID (STOICHIOMETRICALLY EQUIVALENT TO 5 MG. OF PGA) AS SODIUM SALT

SUBJECT	ROUTE OF ADMINISTRATION	PER CENT OF DOSE EXCRETED (MEASURED AS PGA)		EXCRETION IN FIRST 6 HOURS (% OF 24-HOUR EXCRETION)
		0 – 6 HR.	6 – 24 HR.	
2	Oral	21	7	75
3	Oral	37	6	88
5	Oral	33	5	87
8	Oral	42	6	88
2	Oral	11		
3	Oral	42		
5	Oral	30		
8	Oral	43		
2	Intravenous	15	9	62
4	Intravenous	14	15	48
5	Intravenous	14	11	56

as pteroylglutamic acid, was found in the urine during the period between six and twenty-four hours after dosage. The effect was especially marked following intravenous administration.

The last compound studied was the diamide of pteroylglutamic acid.[11] This substance does not appreciably stimulate the growth of *L. casei* or *Str. faecalis* R. No increase in the urinary pteroylglutamic acid level was found in twenty-four hour samples following the oral administration of 10 mg. doses to three subjects. The compound was prepared from *p*-aminobenzoylglutamic acid diamide, 2,3-dibromopropionaldehyde, and 2,4,5-triamino-6-hydroxypyrimidine. Hydrolysis of the compound by autoclaving with 0.4 N NaOH for one hour at 130° C. resulted in the liberation of 69 per cent of the calculated quantity of pteroylglutamic acid, as measured by assay with *Str. faecalis* R.

DISCUSSION

The apparent ability of the body to hydrolyze pteroyldiglutamic acid, as evidenced by the urinary excretion data in the present investigation, is in contrast to the microbiologic inactivity of this substance but may be in harmony with its reported effect in producing a remission in macrocytic anemia.[12] The inactivity of pteroyldiglutamic acid for *L. casei* and *Str. faecalis* R may indicate that these organisms do not possess a "conjugase" system which is effective in hydrolyzing the peptide linkage in pteroyldiglutamic acid between the terminal glutamic acid and the α-carboxyl group of the glutamic acid radicle of the pteroylglutamic acid moiety of the molecule. In contrast, pteroyltriglutamic acid, in which the glutamyl radicles are linked through their γ-carboxyl groups, is fully active for *L. casei* which may mean that this organism has a "conjugase" system which hydrolyzes the γ linkage between glutamyl groups. An alternative explanation would be that *L. casei* utilizes both pteroylglutamic acid and pteroyltriglutamic acid, but not pteroyldiglutamic acid, as precursors of more complex substances.

The nature of the possible breakdown products of pteroylglutamic acid that are excreted in the urine is a matter of speculation. The presence of

xanthopterin in normal urine has been repeatedly noted and it is logical to infer that this substance may be formed by the breakdown of pteroylglutamic acid.*

The present study indicated that pteroyltriglutamic acid is rapidly absorbed following administration, for pteroylglutamic acid was excreted in the urine at approximately the same rate following either oral or intravenous administration of pteroyltriglutamic acid. The data also indicate that, as previously noted,[5] pteroyltriglutamic acid is readily hydrolyzed to pteroylglutamic acid in the body. The site of this breakdown is not known, but preparations made from various organs have been shown to have "vitamin B_c conjugase" activity[13] as indicated by liberation of pteroylglutamic acid from pteroylheptaglutamic acid. Certain enzyme preparations of this type, prepared from chicken pancreas, have been found readily to liberate pteroylglutamic acid from pteroyltriglutamic acid.[14]

The limited data in Table I appeared to indicate that, with a 5 mg. dose of pteroylglutamic acid, an oily vehicle appeared to depress the excretion and hence possibly the uptake of an oral dose; also, the rate of excretion following the administration of a solution of the sodium salt was somewhat higher than when a suspension of the free acid was used.

CONCLUSIONS

1. The urinary excretion of folic acid, as measured by assay with *Str. faecalis* R, was studied with normal subjects receiving various dosages of pteroylglutamic acid (PGA), pteroyltriglutamic acid, and pteroyldiglutamic acid.

2. The proportion of the administered dose of pteroylglutamic acid which was excreted was found to increase as the dose was raised.

3. With pteroylglutamic acid most of the excretion took place within the first six hours, a small part of the administered dose was excreted in the next eighteen hours, and after twenty-four hours the excretion returned to the basal level of between 5 and 10 millimicrograms per milliliter.

4. The microbiologic assay data indicated that pteroyltriglutamic acid was readily hydrolyzed to pteroylglutamic acid in the body when administered by either the oral or intravenous route.

5. Similar results were observed following the administration of pteroyldiglutamic acid. A slight lag in the excretion rate observed with pteroyltriglutamic acid was somewhat more marked in the case of pteroyldiglutamic acid.

6. No increase in the urinary pteroylglutamic acid level was found following the administration of the diamide of pteroylglutamic acid.

The collaboration of Mr. J. Van Meter in assisting with the microbiologic assays is gratefully acknowledged.

*Recently it has been reported by Denko (paper presented at the One Hundred Twelfth Meeting of the American Chemical Society, New York, September, 1947) that, following oral administration of 10 mg., the apparent urinary excretion of pteroylglutamic acid as measured with *L. casei* in some cases far exceeded the dose. No such observation was made in the present study, using *Str. faecalis* R.

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ANOMALOUS FINDINGS IN PENICILLIN LEVEL DETERMINATIONS IN URINE

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INTRODUCTION

NUMEROUS investigators (including Florey and co-workers,[2] Rammelkamp and associates, 1943,[3, 4] Free and co-workers, 1944,[5] Mutch and Rewell, 1945,[6] McDermott and associates, 1946,[7] and Eagle, 1947[8]) have shown that from 60 to 100 per cent of the penicillin administered in a single dose may be voided during the first four hours after injection.

However, in the course of determining penicillin levels in a series of urine specimens obtained from patients under treatment for syphilis, unexpectedly high values were observed in a number of urines collected up to the fourth hour following the administration of penicillin. This report deals with the results obtained from a study of these specimens tested for streptococcidal action at regular intervals over a three-month period. Since these findings were in excess of what could be explained by the dose injected, some of the factors influencing the test also will be discussed.

MATERIALS

The urines tested were sent to the laboratory for routine penicillin assay.* All specimens were refrigerated from time of collection, although a delay of several days until time of assay was frequent. The volume of the samples varied from 50 to 100 milliliters. Unfortunately, the exact volume voided at one time was recorded in only a few instances.

METHODS

Assays were made by the Rammelkamp method.[9] The samples were filtered through a Seitz filter immediately upon reception at the laboratory, to insure sterility. Before assay, appropriate preliminary dilutions of the urine were made in phosphate buffer pH 6.0, so that an end point would be obtained by the use of not more than fifteen serial dilutions.

The test organism was a Group A hemolytic streptococcus, Strain 98.† This strain was regularly sensitive to between 0.0039 and 0.0078 Oxford units of penicillin per milliliter.

The sample and culture dilutions were made in beef heart broth pH 7.0 to which 50 mg. per liter of para-aminobenzoic acid had been added. The tests and dilution tubes were kept in an ice bath during the execution of the test.

EXPERIMENTAL RESULTS

None of the specimens of urine tested before treatment was initiated showed streptococcidal action equivalent to more than 0.04 unit of penicillin.

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*By Dr. Alfred Cohn, Venereal Disease Research Division.

†Obtained through the courtesy of Dr. Rammelkamp.

Furthermore, 249 specimens collected from 5 to 143 hours following drug administration showed both reproducible and reasonable assay values. However, these conditions did not prevail in many specimens collected up to the fourth hour following treatment. Anomalous findings were first observed in this group of specimens in a series of six individuals tested in May, 1946. In the initial assay seven of twelve specimens showed levels from five to one thousand times higher than was anticipated. During a period of five weeks' storage another seven of twelve specimens showed an increase in titer from ten to one hundred fold over the initial test. Only one specimen failed to show increased bactericidal action at some time during this period. These results were observed when the subjects were given penicillin in saline, as well as in water and oil emulsion.

Tables I and II show typical protocols. Patient D. B. (Table I) received one injection of 150,000 Oxford units of penicillin in saline solution. Specimens were collected two, four, eight, twelve, and twenty-four hours following the administration of the antibiotic. These samples were tested at weekly intervals over a period of one hundred days. In instances where more than one test was made during a given period of time, the figures represent an average of the results. All figures represent units per milliliter. Each individual specimen contained between 50 and 100 milliliters. The two-hour sample showed a recovery of 4,000 units of penicillin per milliliter. This would mean that the entire sample contained between 200,000 and 400,000 Oxford units, which was more than the amount administered. Furthermore, during the third week of storage, assays showed that the level per milliliter was equivalent to the entire dose injected, and the level increased tenfold more during the following three weeks. Subsequently it dropped to anticipated levels. Specimens collected on the fourth, eighth, twelfth, and twenty-fourth hour showed regular and reproducible results.

TABLE I. PENICILLIN LEVEL DETERMINATION (PATIENT D. B.; DIAGNOSIS, SYPHILIS; PENICILLIN ADMINISTERED, 150,000 O. U. IN SALINE)

	TIME OF URINE COLLECTION AFTER ADMINISTRATION OF PENICILLIN (HR.)				
DAYS' STORAGE (5° C.)	2 (UNITS RECOVERED/ML.)	4 (UNITS RECOVERED/ML.)	8 (UNITS RECOVERED/ML.)	12 (UNITS RECOVERED/ML.)	24 (UNITS RECOVERED/ML.)
0	--	--	--	--	--
1 to 7	4,000	62	2.0	0.80	0.04
8 to 14	--	--	--	--	--
15 to 21	160,000	250	5.0	0.62	0.04
22 to 40	1,000,000	80	2.5	0.62	0.04
59 to 78	1,300	65	5.0	--	--
100	500	40	2.5	0.31	--

Patient D. (Table II) received one dose of 1,000,000 Oxford units of penicillin in saline solution. Again more antibiotic action than would be expected from the treatment given was found in the two- and four-hour samples. Similar observations were made on tests made during the first five weeks after collection. However, these same specimens showed normal levels from the sixth through the fourteenth week. Tests on the eight-, twelve-, and twenty-four hour specimens again showed regular and reproducible results.

TABLE II. PENICILLIN LEVEL DETERMINATION (PATIENT D.; DIAGNOSIS, SYPHILIS; PENICILLIN ADMINISTERED, 1,000,000 O. U. IN SALINE)

DAYS' STORAGE (5° C.)	TIME OF URINE COLLECTION AFTER ADMINISTRATION OF PENICILLIN (HR.)				
	2 (UNITS RECOVERED/ML.)	4 (UNITS RECOVERED/ML.)	8 (UNITS RECOVERED/ML.)	12 (UNITS RECOVERED/ML.)	24 (UNITS RECOVERED/ML.)
0	--	--	--	--	--
1 to 7	2,000,000+	8,000	125	4.0	4.00
8 to 14	--	--	--	--	--
15 to 21	400,000	400,000+	125	5.0	1.25
22 to 35	2,000,000+	25,000	--	--	--
36 to 60	6,200	800	25	2.5	0.62
61 to 100	5,000	75	40	2.5	--

In October and November, 1946, the one-hour urine specimens of another six subjects who received a single dose of 1,000,000 Oxford units of penicillin in water and oil emulsion were studied. As before, the total volume varied between 50 and 100 ml. per sample. Table III shows the results obtained per milliliter in four typical instances. The general pattern of the early specimens tested in May was repeated. Specimens obtained from these individuals immediately preceding treatment showed no bactericidal activity. Furthermore, the activity of the one-hour specimens was completely destroyed by the use of the penicillinase broth.

TABLE III. UNITS OF PENICILLIN RECOVERED IN URINE COLLECTED FROM SYPHILITIC SUBJECTS ONE HOUR AFTER THE ADMINISTRATION OF 1,000,000 O. U. IN WATER AND OIL EMULSION*

STORAGE (DAYS)	PATIENT			
	1 (UNITS/ML.)	2 (UNITS/ML.)	3 (UNITS/ML.)	4 (UNITS/ML.)
4 to 7	25,000	6,200	340,000	80,000
8 to 14	2,500,000	620,000	--	--
15 to 21	--	--	--	--
22 to 35	12,500	12,500	800	1,600
36 to 100	8,000	2,000	600	700

*Squibb penicillin 12003-1; expiration date, April 1, 1947.

The possibility that certain specimens might contain a substance capable of producing a synergistic effect in the presence of penicillin was then investigated. Accordingly, 10,000 Oxford units of a commercial penicillin per milliliter were added to a 10 c.c. portion of pretreatment urine. This sample was then tested at the same time as a specimen collected from this same subject one hour after treatment was started.

Results of these comparative tests are shown in Table IV. The one-hour specimens again showed potentiated bactericidal activity, while the pretreatment urine to which 10,000 Oxford units of penicillin per milliliter were added showed regular and reproducible results which were similar to the result obtained when an equivalent amount of penicillin was diluted with phosphate buffer at pH 6.0. Again the untreated pretreatment urine showed no bactericidal activity, and the addition of penicillinase to either the one-hour urine sample or the penicillin in buffer solution destroyed the activity of the test sample.

TABLE IV. UNITS OF PENICILLIN RECOVERED FROM PRETREATMENT URINE TO WHICH PENICILLIN WAS ADDED AFTER EXCRETION COMPARED WITH LEVELS FOUND IN URINE OF THE SAME PATIENT COLLECTED ONE HOUR AFTER ADMINISTRATION OF 1,000,000 O. U. IN WATER AND OIL EMULSION*

	TIME OF STORAGE AT 5° C.				
	0 DAYS (UNITS RECOVERED/ML.)	1 TO 7 DAYS (UNITS RECOVERED/ML.)	8 TO 14 DAYS (UNITS RECOVERED/ML.)	15 TO 21 DAYS (UNITS RECOVERED/ML.)	22 TO 35 DAYS (UNITS RECOVERED/ML.)
Test Fluid					
Urine collected one hour following the administration of 1,000,000 O. U. in water and oil emulsion	--	25,000	2,500,000	12,500	8,000
Pretreatment urine + 10,000 O. U./ml.	10,000	10,000	10,000	10,000	10,000
Controls					
Buffer pH 6.0+ 10,000 O. U. penicillin/ml.	10,000	10,000	10,000	10,000	10,000
Untreated pretreatment urine	0	0	0	0	0
One-hour urine + penicillinase	0	0	0	0	0
Penicillin in buffer pH 6.0 + penicillinase	0	0	0	0	0

*Squibb penicillin 12005-1; expiration date, April 1, 1947.

It may be considered that these results were caused by a technical error in the method of dilution of the test samples. Experiments were therefore set up using different initial dilutions, different dilution volumes, and different diluents. No significant variations were observed.

To ascertain whether the high levels were caused by experimental accidents, twenty-four different one-hour specimens* were divided each in three aliquot parts and tested at regular intervals for a period of six weeks. Again, there was never more than a twofold variation between the different portions of any one specimen.

It was thought that the delay of twenty-four or more hours between the time of collection of the specimens and the time of assay might be a contributing factor in producing the phenomenon of enhanced bactericidal activity. To investigate this possibility, the previously mentioned twenty-four specimens collected one hour after the administration of 1,000,000 O.U. of penicillin in Pendil-beeswax were filtered and tested within one hour after excretion. All tests were carried out in triplicate over a six-week period. During this period there were no significant changes in titer in any of the twenty-four samples.

COMMENTS AND CONCLUSIONS

In a study of sixty-seven early specimens of urine anomalous findings have been observed in thirty instances. These results did not appear to be caused by the usual technical errors in experimental accidents. Furthermore, the

*Twenty-one specimens from patients receiving 1,000,000 O.U. Heyden penicillin #1276 (expiration date, Aug. 1, 1948) diluted in Pendil-beeswax; three specimens from patients receiving 1,000,000 O.U. Parke Davis penicillin #02314A (expiration date, July, 1948) diluted in Pendil-beeswax.

phenomenon of increased bactericidal action was not observed in any of the 249 specimens collected more than four hours after treatment.

No explanation for this phenomenon has been found. It is reported, at this time, to call attention to irregularities that may be encountered in attempting to make a quantitative estimate of the penicillin content of early urines.

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THE ANTIBACTERIAL ACTIVITY OF SOME SULFON- AND SULFANILANILIDE DERIVATIVES

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Rensselaer, N. Y.

AN EARLIER communication[1] from these laboratories presented the general antibacterial activity of a series of benzenesulfonic acid derivatives. The most effective compound there referred to was found to be 3′,5′-dibromosulfanilanilide or the corresponding dichloro derivative. The removal of the amino group resulted in only a slight loss in activity. Several monohalogenated and dihalogenated compounds were tested in which the bromine or chlorine atoms were placed in various positions on the anilide portion of the molecule. Antibacterial activity was practically lost when a halogen was placed in the 2-position. While all these compounds displayed definite antibacterial action, none approached that of 3′,5′-dibromosulfanilanilide. The latter compound was also tested for antibacterial properties against pathogens, most of which cause infections which do not readily respond to present chemotherapeutic agents.[2]

In order to obtain a better correlation between chemical structure and degree of antibacterial action, it seemed worth while to prepare additional benzenesulfonic acid derivatives. Accordingly, the following compounds* were tested by the present authors.

COMPOUND	NAME	MOL. WEIGHT
1	2′,6′-dichlorosulfanilanilide	302.2
2	3′,5′-dibromo-4′-hydroxybenzenesulfonanilide	407.0
3	3′,5′-dibromo-4′-hydroxysulfanilanilide	422.1
4	3′-bromo-5′-chloro-4′-hydroxysulfanilanilide	377.6
5	3′-bromo-5′-chlorosulfanilanilide	361.6
6	3′,5′-diiodo-4′-hydroxysulfanilanilide	516.0
7	3′,5′-diiodosulfanilanilide	500.0
8	3′,5′-bis-(trifluoromethyl)-sulfanilanilide	384.3
9	3′-trifluoromethylsulfanilanilide	316.3
10	3′,5′-dibromo-o-aminobenzenesulfonanilide	406.1
11	3′,5′-dibromo-m-aminobenzenesulfonanilide	406.1
12	N^4succinyl-3′,5′-dibromosulfanilanilide	506.2
13	3′,5′-dibromosulfanilanilide	406.1

METHODS

The method for testing the antibacterial action of the compounds was essentially the same as that outlined in the previous report.[1] This consisted of dissolving the drugs in a few c.c. of alcohol preparatory to making an initial dilution in nutrient broth. Serial dilutions were prepared in broth from 1:1,000 up to and including 1:1,024,000. After autoclaving, the drug-broth mixtures were inoculated with a test organism and incubated at 37° C. for seventy-two hours. Tubes containing no growth or growth less than one-half that of the drug-free control after incubation for twenty-four hours (forty-eight hours in test with *Actinomyces bovis*) were considered to contain a bacteriostatic concentration of drug. All

From the laboratories of the Winthrop Chemical Co., Inc.
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*Prepared by Dr. A. Mooradien of the Sterling Winthrop Research Institute.

TABLE I. HIGHEST DILUTION OF C[illegible]NDS E[illegible]

COMPOUND	PNEUMOCOCCUS TYPE I		STREPTOCOCCUS PYOGENES-C203		STREPTOCOCCUS FAECALIS		STREPTOCOCCUS VIRIDANS		STAPHYLOCOCCUS AUREUS		NEISSERIA GONORRHOE[illegible]		BRUCE ABORT
	BS*	BC	BS	BC	BS	BC	BS	BC	BS	BC	BS	BC	BS
1	2	<1	<1	<1	–	–	–	–	2	<1	–	–	32
2	8	4	4	2	4	<1	4	1	32	1	16	8	8
3	32	8	2	2	16	<1	2	<1	4	<1	8	8	32
4	32	16	4	2	16	<1	4	<1	8	<1	16	16	8
5	128	64	64	32	16	<1	16	8	128	1	32	32	16
6	32	16	4	2	8	<1	4	<1	16	<1	8	8	4
7	64	64	32	16	32	<1	32	<1	128	<1	256	64	64
8	256	128	64	16	16	<1	16	16	512	128	64	64	128
9	64	32	64	16	4	2	8	4	16	4	32	16	32
10	32	2	128	<1	<1	1<	<1	<1	128	<1	256	128	128
11	64	32	16	16	8	<1	16	<1	512	<1	32	32	64
12	4	2	4	2	1	<1	2	1	32	1	8	4	8
13	128	64	32	32	<1	1<	16	8	64	<1	64	32	64

*Figures represent dilutions bacteriostatic (Bs) or bactericidal (Bc) as expressed in thousands, th[illegible] is, 128 = 128,000.

<1, Concentrations greater than 1:1,000 not tested; —, not tested.

tubes in which growth failed to appear after incubation for seventy-two hours were subcultured into fresh drug-free broth, and the subculture tubes incubated for an additional seventy-two hours. Failure of growth to develop in the latter was taken as evidence of a bactericidal action in the tubes from which they were subcultured. Bacteriostatic and bactericidal action against the gonococcus was determined by subculturing on dextrose-starch agar.* In the study, 3′,5′-dibromosulfanilanilide (Compound 13) was included for purpose of comparison. The results of this investigation are presented in Table I.

RESULTS

The only compound which appears to approach or to exceed the activity of 3′,5′-dibromosulfanilanilide (compound 13) is a derivative containing a large number of fluorine radicals (compound 8). The iodo analogue (compound 7) of compound 13 and the 5-bromo-3-chloro derivative (compound 5) appear to exert an activity comparable to that of the parent compound. The introduction of the hydroxyl group on the 4-position of the anilide invariably results in a decrease of activity (compare compound 3 with 13, 4 with 5, and 6 with 7). The succinyl derivative (compound 12) also displays a marked decrease in antibacterial action.

The ortho- and metaisomers (compounds 10 and 11) present an action equal to that of 3′,5′-dibromosulfanilanilide when all the test organisms are collectively taken into consideration. This would tend to indicate that a free amino group in the para-position is not essential for maximum activity, and may thus explain why the action of 3′,5′-dibromosulfanilanilide against *Streptococcus pyogenes* is unaffected by the presence of p-aminobenzoic acid.

SUMMARY

Several new derivatives of 3′,5′-dibromosulfanilanilide have been prepared and tested for antibacterial action in vitro against type I pneumococcus, *Streptococcus pyogenes* (C203), *Streptococcus faecalis*, *Streptococcus viridans*,

*Difco Laboratories, Inc., Detroit, Mich.

...UNDS EXHIBITING ANTIBACTERIAL ACTION

BRUCELLA ABORTUS		HEMOPHILUS DUCREYI		PASTEURELLA PESTIS		VIBRIO CHOLERAE		CLOSTRIDIUM WELCHII		ACTINOMYCES BOVIS	
BS	BC	BS	BC	BS	BC	BS	BC	BS	BC	BS	BC
32	<1	–	–	–	–	–	–	<1	<1	–	–
8	2	16	1	32	2	16	2	16	2	64	2
32	1	16	1	32	2	64	4	4	1	16	1
8	1	16	<1	64	2	32	2	4	1	4	<1
16	8	16	8	128	16	32	8	8	2	128	16
4	<1	16	1	16	2	32	8	8	2	16	1
64	<1	64	16	128	<1	32	<1	64	2	256	16
128	<1	32	<1	32	<1	32	<1	64	32	128	32
32	4	16	2	32	4	16	4	8	4	32	8
128	<1	<1	<1	2	<1	1	<1	4	1	256	64
64	<1	8	<1	64	1	32	<1	16	16	64	32
8	1	2	<1	8	1	8	1	2	1	8	1
64	16	512	32	64	2	32	1	32	16	128	16

Staphylococcus aureus, Neisseria gonorrhoeae, Clostridium welchii, Brucella abortus, Hemophilus ducreyi, Pasteurella pestis, Vibrio cholerae, and *Actinomyces bovis* (anaerobic).

The 3′,5′bis-(trifluoromethyl) derivative was found to be somewhat superior to 3′,5′-dibromosulfanilanilide, while the 3′,5′-diiodo and the 3′-bromo-5′-chloro derivatives were about equal to 3′,5′-dibromosulfanilanilide in antibacterial action.

The ortho- and metaisomers in which the NH_2 group is not in the para-position display an activity equal to 3′,5′-dibromosulfanilanilide, while the hydroxysulfanilanilide and succinyl derivatives which were tested were found to be less active than the latter compound.

Since this paper was prepared, Schmidt and Sesler[3] demonstrated that 3′,5′-dibromosulfanilanilide and a series of derivatives therefrom held little promise as effective chemotherapeutic agents in bacterial infections. McChesney[4] more recently described the toxicity, absorption, and metabolism of 3′,5′-dibromosulfanilanilide which account for its relative ineffectiveness in infection-protection tests in experimental animals.

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THE EFFECT OF 2,3-DIMERCAPTOPROPANOL (BAL) ON THE TOXICITY OF GOLD

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BAL (2,3-dimercaptopropanol) was first introduced as an agent capable of combating the arsenic toxicity which results from Lewisite burns.[1] In addition to reducing the toxicity of compounds of arsenic,[2] this sulfhydryl compound has been shown to diminish the toxicity of antimony,[3] mercury,[3] chromium,[3] bismuth,[3] and nickel.[4]

Gold compounds have found rather widespread clinical use in the treatment of rheumatoid arthritis. However, due to the toxicity of this heavy metal, extreme caution is required in its therapeutic use. The purpose of this study was to determine in experimental animals whether some of the gold compounds commonly used as therapeutic agents could be rendered less toxic by the use of BAL.

While this work was in progress there appeared a series of papers covering the effect of BAL on the toxic symptoms produced by gold in human subjects.[5-7] Ragan and Boots[6] mentioned that BAL reduces the toxicity of gold in rats, but few data were presented.

METHODS

For acute toxicity studies, groups of adult hooded rats, varying in weight from 190 to 326 grams, were given one of the gold compounds studied. All administrations were made intramuscularly and the survival time was recorded. The dose of the gold compound administered was that which was determined by preliminary trial to cause death within a few days. One control group of animals received only the gold compound. A second (test) group received the same dose of the same gold compound in one thigh and BAL in the muscle of the opposite leg. Additional groups of rats received the same dose of gold, but the BAL was administered in multiple doses. Any change in the survival time was considered to be due to the modification of the toxicity of the gold compound tested.

In the studies of the effect of gold and BAL on the rate of growth of young rats, all the animals except those which received neither gold nor BAL were litter mates. In this portion of the experiment the animals received a single sublethal dose of gold and also a single dose of BAL. Weights usually were recorded every second day during the early portion of this work; later a longer time interval was used. Growth was recorded for a period of twenty-three days. At the end of this period the animals were sacrificed and portions of the liver and kidney were fixed in formalin for microscopic examination.

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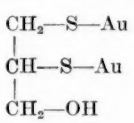
The toxicities of the following gold-containing compounds were studied: gold sodium thiosulfate, gold sodium thiomalate (Myochrysine*), sodium succimidoaurate,† and gold thioglucose.‡ The last substance was supplied both as a pure solid and as a suspension in an oily medium. A commercial 10 per cent solution of BAL in oil and benzyl benzoate was used.

It was found that if an aqueous solution of BAL was added to an aqueous unbuffered solution of either gold sodium thiosulfate or gold sodium thiomalate a heavy yellow precipitate formed immediately. In the case of sodium succimidoaurate and gold thioglucose no precipitate formed on mixing solutions of these compounds with aqueous BAL. The BAL-gold compound proved to be insoluble in water, dilute HCl or NaOH, alcohol, and ether. The BAL-gold compound formed with either gold sodium thiomalate or gold sodium thiosulfate was washed successively with water, alcohol, and ether, and dried in a vacuum dessicator over sulfuric acid. The content of gold in each sample was determined by ashing according to the method of Pregl[8] and by weighing the gold residue. In addition, a sample of the BAL gold compound containing two atoms of gold per molecule of BAL was prepared according to the procedure of Weiss[9] by adding one mol of gold bromide to 0.8 mol of BAL. The analyses of the compounds are given below:

BAL-gold compound from gold sodium thiosulfate	72.6 per cent Au
BAL-gold compound from gold sodium thiomalate	73.7 per cent Au
BAL-gold compound from gold bromide	73.6 per cent Au
Theoretical $C_3H_6O_3SAu_2$	76.4 per cent Au

These analyses indicate that the compounds formed are about 95 to 96.5 per cent pure. The probable structure for the BAL-gold compounds is:



RESULTS

Gold sodium thiosulfate was found to be a toxic compound. Six groups of animals were studied. The results of this experiment are summarized in Table I. Rats given this compound in doses equivalent to 75 mg. of gold per kilogram usually died within six hours. The administration of 12.5 mg. of BAL just before the gold sodium thiosulfate appeared to reduce the toxicity of the gold compound by about one-third. The survival time of the rats was the same whether a single dose of BAL was given either one minute before or one hour after the gold. If two doses of BAL were given, one a minute after the gold and the second five hours later, there was a prolongation of the survival time as compared with a single dose of BAL. Four of the ten animals in this series survived the test period of fourteen days and then were sacrificed. If three doses of BAL were administered at 1-minute, 3-hour, and 6-hour intervals after the gold, the effects were in general similar to those obtained with two doses of BAL. However, only two of the animals in this group survived the period of study. The animals which survived only about six hours probably died of respiratory and vasomotor paralysis.[10] The animals which lived three to six

*Merck & Co., Inc., Rahway, N. J.

†This compound was supplied through the courtesy of Inventions, Inc., Chicago, Ill.

‡Both Solganal-B Oleosum (gold thioglucose) and the pure gold thioglucose were supplied by the Schering Corporation, Bloomfield, N. J.

TABLE I. EFFECT OF BAL ON THE TOXICITY OF GOLD COMPOUNDS; BOTH BAL AND GOLD COMPOUNDS ADMINISTERED SUBCUTANEOUSLY

SUBSTANCE	DOSE OF GOLD (MG./KG.)	BAL ADMINISTERED			SURVIVAL TIME (DAYS)
			TIME		
		MG.	BEFORE Au	AFTER Au	
Gold sodium thiosulfate	30	0			2,2,2,3
	75	0			1/6,1/4,1/4,1/4,1/4,1/4,1/4,1/4,1/2,1/2
	75	12.5	1 min.		2,2,2,2,4,4
	75	12.5		1 hr.	3,3,3,4
	75	12.5		1 min.	
		12.5		5 hr.	2,3,5,5,5,6, s,*s,s,s
	75	12.5		1 min.	
		12.5		3 hr.	
		12.5		6 hr.	3,3,3,4,4,4,6, s,s
Gold sodium thiomalate	75	0			3,4,4,5,6,6, s,s
	75	12.5		1 min.	3,3,4,5,5,5
	100	0			3,3,4,4,4,4
	100	12.5		5 min.	2,2,4,5,5,7
	100	12.5		3 hr.	
		12.5		24 hr.	3,4,5,5
	100	12.5		1 min.	
		12.5		4 hr.	
		12.5		7 hr.	2,2,2,3,4,5
Gold thioglucose	75	0			s,s,s,s,s,s
	100	0			3,3,4,4,4
	100	12.5		1 min.	2,3,4,6,8,9
	100	12.5		1 min.	
		12.5		3 hr.	
		12.5		6 hr.	s,s,s,s,s,s,s
Sodium succimidoaurate	75	0			3,3,3,3,4,4
	75	12.5		1 min.	s,s,s,s,s,s

*s, The animal survived for the experimental period of fourteen days.

days showed large soft, grayish spotted kidneys on death. Microscopic examination of the kidney showed extreme damage to the tubules of this organ. Renal insufficiency was believed to be the cause of death.

In the study of acute toxicity, gold sodium thiomalate was administered to six groups of animals. The results of these experiments also are summarized in Table I. The amount of gold used in the first group of animals in this series was equivalent to that used in the gold sodium thiosulfate study (75 mg. per kilogram). Survival time for the animals treated with gold sodium thiomalate was considerably longer than that observed with gold sodium thiosulfate. The administration of BAL immediately after giving gold sodium thiomalate did not appear to have any effect on the survival time of the animals. In the remainder of this study, the dose of gold sodium thiomalate was increased to the equivalent of 100 mg. of gold per kilogram in order to decrease the survival time of the animals. Thus, any detoxifying effect of BAL could be more easily detected. The administration of 12.5 mg. of BAL to animals five minutes after the dose of gold appeared to have no effect on the survival time. If two doses of BAL were given, one three hours and the other twenty-four hours following the gold, there was no prolongation of the survival time as compared with the control animals of this series. If three doses of BAL were given, one immediately after the gold, the second four hours later, and the last seven hours after the gold, there was a slight decrease in the length of their survival. This effect may have been due to the sum of the toxicities of BAL and gold.

The toxicity of sodium succimidoaurate can be effectively reduced by the administration of BAL after the gold compound. Table I shows that, following administration of the BAL, there were no deaths due to gold.

An attempt was made to produce death in rats by administering gold thioglucose which was supplied in an oil media, but the dose required to produce death consistently was too high to have value in this study. Four of the rats received as much as 2 ml. of the oily suspension which contained 200 mg. of gold (approximately 800 mg. per kilogram). Two of the animals survived and two died, one on the third and one on the fifth day after injection. At autopsy, the animal that lived only three days had a large mass of yellow oil at the site of injection, showing that the absorption of the gold compound was poor. On the other hand, if an aqueous solution of gold thioglucose was administered in a dose supplying 100 mg. of gold per kilogram, the animals usually died within four days. Three doses of BAL completely protected the animals, as shown by survival of all seven animals tested.

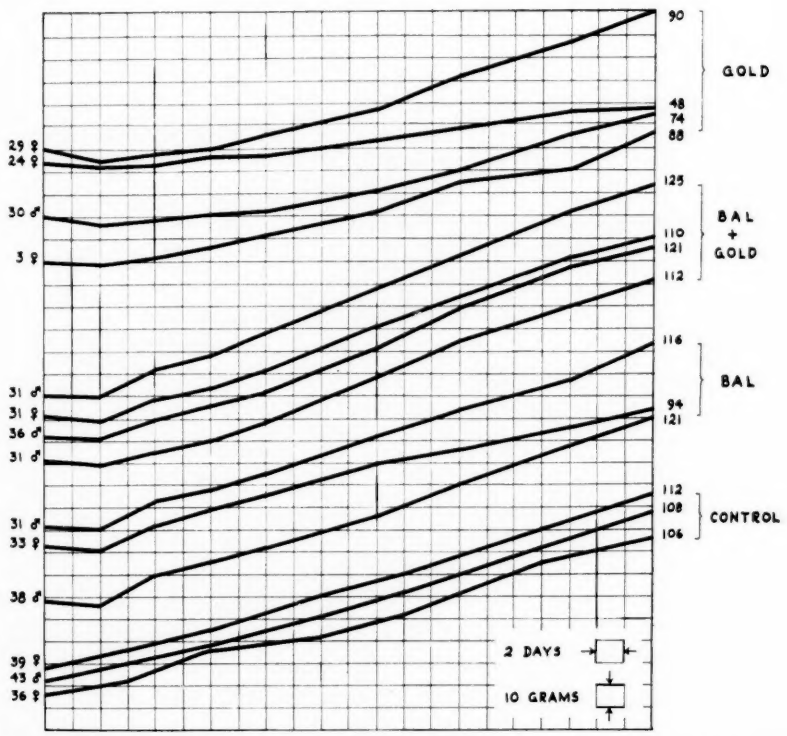


Fig. 1.—The effect of a single dose of gold sodium thiomalate on the growth of young rats. The dose of gold was 50 mg. per kilogram and that of BAL 3.1 mg. per animal. These compounds were given intramuscularly, at the start of the experiment. The numbers along the left border are the initial weight in grams and those on the right are the final weight twenty-three days later.

The effect of BAL on the rate of growth of rats given a nonlethal dose of gold (50 mg. per kilogram) was considered a more sensitive test than the survival time. The young rats which received the single dose of gold sodium thiomalate had rather shallow growth curves, while those which received the

same dose of gold plus BAL (3.1 mg.) had growth curves of approximately the same slope as those which received no treatment or only BAL (Fig. 1). Microscopic examination of the livers and kidneys of these animals was made. The kidneys of those animals which received only the gold showed "marked cloudy swelling of the proximal convoluted tubules with karyolytic and karyorrhectic nuclear changes and plugging of the tubules with necrotic debris some of which contains calcium. There are evidences of regenerative epithelial hyperplasia of the cells of these tubules. There is marked cloudy swelling and nuclear degeneration of scattered liver cells."* Those which received the gold followed by the BAL showed "moderate cloudy swelling of the cells of the proximal convoluted tubules. A few basophilic hyaline casts are seen."* BAL alone produced a similar picture.

DISCUSSION

In this study of the effect of BAL on the toxicity of various gold compounds, it is of interest that the gold compounds were of different chemical classes. The gold sodium thiosulfate contained monovalent gold bound in an inorganic linkage. Gold sodium thioglucose and gold sodium thiomalate contained monovalent gold bound to an organic radical. The attachment of the gold to the carbon chain was through a sulfur linkage. Sodium succimidoaurate contained trivalent gold bound in a sulfur-free organic compound.

The data presented in Table I show that, under the conditions described, BAL reduces the toxicity of gold with all compounds tested except gold sodium thiomalate. It should be noted that the doses of gold used in the acute toxicity studies were relatively high. If smaller doses of gold sodium thiomalate were given and growth (as measured by an increase in weight) was used as a criterion of toxicity, BAL effectively detoxified gold sodium thiomalate.

When BAL was given with large doses of gold sodium thiosulfate the animals usually survived the factors which produced rapid death, but some of them later succumbed to the nephrotoxic effect of the heavy metal. This action also has been noted where the effect of BAL on cadmium poisoning was studied.[1] The explanation given was that the BAL-metal compound is reabsorbed by the kidney tubule and produces toxic action there.

SUMMARY

The effect of BAL on the survival time of rats given subcutaneous injections of lethal doses of gold, as gold sodium thiosulfate, gold sodium thiomalate, gold thioglucose, or sodium succimidoaurate was studied. BAL caused an increase in survival time with all compounds studied except gold sodium thiomalate. With sublethal doses of gold sodium thiomalate, BAL has a definite effect in reducing toxicity of this heavy metal when growth was used as a criterion.

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*The interpretations of the tissue changes in this study were by Dr. J. L. Chason of the Department of Pathology of the Wayne County General Hospital, Eloise, Mich.

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HISTAMINE ANTAGONISTS

VIII. N-(α-Pyridyl)-N-(α-Thienyl)-N′,N-Dimethylethylenediamine, a New Antihistaminic Compound. Experimental and Clinical Experiences

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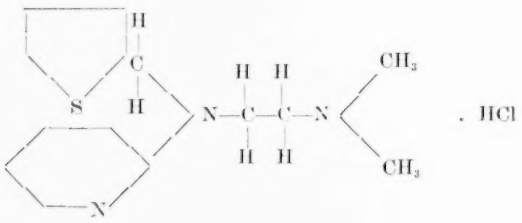
Chicago, Ill.

A NEW antihistaminic drug of the ethylenediamine series, N-(α-pyridyl)-N-(α-thienyl)-N′,N-dimethylethylenediamine hydrochloride, made available to us about a year ago,* has been found to possess potent clinical and pharmacologic activities. This substance differs from pyribenzamine in the substitution of a thienyl for the benzyl ring and has the following structural formula[1]:



EXPERIMENTAL FINDINGS

This substance is similar to most of the other antihistaminic drugs in its local anesthetic action. It was found to be a potent antihistaminic substance. Death in guinea pigs from an intravenous M.L.D. 100 dose of histamine† (0.4 mg. histamine base per kilogram) could be prevented in 100 per cent of the animals by the intraperitoneal injection thirty minutes previously of 0.1 mg. of the drug per kilogram. A dose of 0.05 mg. per kilogram protected 50 per cent of the animals. Roth and associates[2] found that 5 mg. of the drug per kilogram intraperitoneally was effective in protecting guinea pigs against 60 M.L.D. 100 of histamine, while Ercoli and associates[3] obtained protection against 150 to 200 lethal doses of histamine by using 10 to 15 mg. of the drug per kilogram.

Roth and associates report that 0.002 γ per cubic centimeter produces 75 per cent or greater inhibition of the contraction of the guinea pig ileum from 0.005 γ per cubic centimeter of histamine base. The activity of this drug in the inhibition of the ileal contraction, as compared with other drugs, is esti-

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*The drug was submitted to us by the research departments of Eli Lilly & Co., Indianapolis, Ind. (Histadyl), and of Abbott Laboratories, North Chicago, Ill. (Thenylene), without either firm being cognizant until several months later that both had synthesized the same compound.

†Histamine diphosphate supplied through the courtesy of Burroughs Wellcome & Co., Inc., New York, N. Y.

mated by Lee and associates[4] as follows: benadryl, 1; pyribenzamine, 4.9; and the thienyl compound, 3.9. Such active inhibition also was found by Ercoli and associates[3] and by us.

Protection against death from aerosolized histamine was studied by Lee and associates, who found the drug six times more potent than benadryl and less than half as potent as pyribenzamine. Ercoli and associates[3] also reported effective protection against histamine aerosol.

Our work along this line was somewhat different. Because of our special interest in the possible therapeutic value of aerosols of antihistamine drugs in asthma, we wished to study the effect of such drug aerosols as protection against bronchospasm from aerosolized histamine. In order to approximate conditions more closely resembling those in human asthma we selected unquestioned labored breathing, rather than death, as the end point in the guinea pig. The animals were exposed for five minutes to a standard aerosol of the drug in one chamber, and after a period of fifteen minutes they were exposed to the standard histamine aerosol. The time elapsing before the onset of dyspnea was noted. By varying the concentrations of the drug aerosol on different days in the same series of animals a fair estimate of the protective ability of the drug could be made. Many experiments with many variations were performed and comparative studies of all available drugs were made. The complete details will be reported at another time. Here we wish to note briefly that moderate concentrations of the drug aerosol (½ to 2 per cent) for five minutes at 5 pounds pressure with a particular atomizer were effective in giving appreciable protection against histamine aerosol (½ mg. of base per cubic centimeter at 5 pounds pressure). This protection was not quite as good as that obtained with pyribenzamine, but better than that obtained with benadryl.

The intraperitoneal injection of 1 mg. per kilogram of this drug in guinea pigs was effective in protecting ten of ten animals against anaphylactic death, while a large control series showed a mortality of 61 per cent.

CLINICAL FINDINGS

The thienyl compound has been found by us to be an effective remedy in the symptomatic relief of allergic manifestations. In 112 patients with seasonal hay fever (including that due to the pollen of trees, grasses, and weeds, and to the spores of molds) seventy-nine, or 70 per cent, were benefited. Of ninety-five patients with nonseasonal vasomotor rhinitis, forty-four, or 46 per cent, received some measure of relief. The dyspnea of asthma was not appreciably altered in thirty patients, although the preasthmatic, spasmodic cough was decidedly helped in six of nine patients. The subjective symptoms of urticaria were helped in seven of twelve patients. In thirteen patients with atopic dermatitis eight obtained considerable relief from the itching. Seven of nine patients with dermographism and one with pruritus of unknown origin were benefited. Pruritus ani was substantially relieved in two of three patients. The experimental wheal produced by histamine or by a specific antigen on the human skin was diminished by the local application of the drug.

As with other previously described antiallergic drugs with antihistaminic action,[5, 6] the relief was only palliative, lasting two to six hours, was not complete, since all manifestations of the allergic ailment were not abolished, and was not obtained in all allergic patients. From our experience thus far, we gain the impression that this drug is an effective agent in the alleviation of allergic manifestations, particularly allergic rhinitis and itching dermatoses. Although the incidence, and perhaps the degree, of relief is not quite as high as that obtained with pyribenzamine, the thienyl compound has the common attribute of other antihistaminic compounds in its selective superiority for particular individuals. In other words, in some patients this drug is more effective than other drugs with a higher therapeutic index.

The average dose of the drug is 50 mg. orally, one to four times daily, depending on the nature of the manifestations. In a few patients we employed 100 mg. doses, but these were frequently not well tolerated. In 253 patients, most of whom received 50 mg. doses, undesirable side actions were noted in sixty-three, or 25 per cent. Sedation was the most common finding, occurring in forty-eight, or 19 per cent, of the patients. The degree of sedation was not as great as that produced by benadryl, but approximated or perhaps exceeded that experienced with pyribenzamine. Our limited use of 100 mg. doses gives us the impression that marked sedation is probably more common than with similar doses of pyribenzamine. Here again, as with therapeutic individuality, we found individuality in sedative effects. We encountered patients who experienced sedation from pyribenzamine and no sedation from the thienyl compound. Other but less frequent side actions noted were vertigo, nervousness, dryness of the mouth and throat, excitation, insomnia, headache, nausea, and diarrhea.

SUMMARY

1. A new antihistaminic compound, N-(α-pyridyl)-N-(α-thienyl)-N′,N′-dimethylethylenediamine hydrochloride, is a potent agent in the prevention of death from histamine injection or aerosol in the guinea pig, in the inhibition of the histamine contraction of the guinea pig ileum, and in the prevention of anaphylactic shock.

2. Aerosols of this drug are capable of preventing bronchospasm and dyspnea in guinea pigs from aerosolized histamine.

3. This drug has a high incidence of effectiveness in the symptomatic relief of some allergic conditions, particularly allergic rhinitis (seasonal and nonseasonal), urticaria, and atopic dermatitis.

4. Side reactions are fairly common and consist mainly of sedation. In degree and incidence sedation is less than that obtained with benadryl and equals or exceeds that of pyribenzamine.

5. The general experimental and clinical efficacy of this drug approaches but does not equal that of pyribenzamine. However, its availability is an advantage because in selected persons it is more effective or less disturbing than pyribenzamine.

6. Neither this nor any other antihistaminic drug has any but palliative function and does not replace the more basic and more lasting benefit obtained from allergic management by avoidance or desensitization.

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EFFECT OF ALKALOIDS ON ACETYLCHOLINE AND POTASSIUM SENSITIVITY OF STRIATED MUSCLE

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IN A study dealing with the mechanism through which muscle shortening is induced, data about agents that modify the induction of muscle shortening may be essential.

In the following, the effects of various concentrations of different alkaloids on the shortening of striated muscle induced by acetylcholine and potassium were investigated.

EXPERIMENTAL

Effect of Alkaloids on Acetylcholine Sensitivity of Striated Muscle.—

Method: The rectus abdominis muscle of the frog was excised and suspended in a muscle chamber containing 10 c.c. frog Ringer's solution. Shortening of the muscle was induced throughout the experimental procedure by immersion in Ringer's solution containing acetylcholine bromine (50 μg per 100 c.c.) for two minutes. Between two immersions in acetylcholine the muscle was washed with Ringer's solution for ten minutes. This procedure was repeated until three successive immersions in the solution of acetylcholine gave similar responses. The shortening of muscle was registered by an isotonic lever on a kymograph. After this stabilization, between two shortenings induced by the acetylcholine solution, instead of washing with Ringer's solution for ten minutes, the muscle was washed for five minutes and was immersed for five minutes in Ringer's solution containing one of the alkaloids to be investigated. Solutions containing the alkaloids in increasing concentrations were used. The pH of all solutions was corrected to 7.

Control: Muscles were immersed only in Ringer's solution and shortening was induced with the acetylcholine solution as described. The repeatedly induced shortenings, each lasting two minutes, were of the same magnitude for at least three hours. This period of time was longer than the duration of the experiments described.

Calculation: The amount of shortening of muscle after immersion in the various solutions to be tested was expressed as a percentage of the amount of shortening of the same muscle induced by acetylcholine before immersion in the solution of alkaloids. All results deviating from 100 per cent by more than twice the square root of the sum of the squares of the standard error of mean of the controls and the standard error of mean of the experiments were considered significant [$2\sqrt{\text{S.E.}^2\text{ (control)} + \text{S.E.}^2\text{ (experiments)}}$].

Results: The effects of the alkaloids on the shortening of the rectus abdominis muscle induced by acetylcholine are given in Table I. The muscle shortening increased in the presence of physostigmine, colchicine, epinephrine, ergotamine, pilocarpine, morphine, and yohimbine (the latter three in higher concentrations). The other alkaloids used decreased the acetylcholine sensitivity of the muscle.

Effect of Alkaloids on Potassium Sensitivity of Striated Muscle.—In the following experiments the effect of the alkaloids on the shortening of muscle

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induced by potassium was investigated to ascertain whether the alkaloids modify the sensitivity of muscle to chemical stimuli other than acetylcholine.

Method: Shortening of muscle was induced by a 20 mM KCl solution instead of acetylcholine, and the effect of the substances on the potassium sensitivity of the muscle was observed as described previously.

Results: The results are given in Table I. The shortening of muscle induced by potassium increased in the presence of all alkaloids (except epinephrine and cocaine), veratrine and colchicine being the most effective agents. The potassium sensitivity decreased in the presence of epinephrine and cocaine and in the presence of higher concentrations of d-tubocurarine, strychnine, quinine, quinidine, and atropine.

TABLE I. EFFECT OF ALKALOIDS ON THE SHORTENING OF THE RECTUS ABDOMINIS MUSCLE INDUCED BY ACETYLCHOLINE AND POTASSIUM

	AMOUNT OF CONTRACTION (IN PER CENT OF CONTROL*); SHORTENING INDUCED WITH											
	ACETYLCHOLINE						POTASSIUM					
	CONCENTRATIONS OF THE SUBSTANCES USED (MG. PER 100 C.C. RINGER'S SOLUTION)											
SUBSTANCE	100	10	1	0.1	0.01	0.001	100	10	1	0.1	0.01	0.001
Atropine	24	34	88	107	108	107	79	114	109	113	107	103
Amphetamine	23	74	92	98	99	98	88	108	110	134	132	122
Cinchonine	---	11	79	96	94	102	---	117	116	112	113	99
Cocaine	---	s†	77	97	101	102	---	s	77	85	90	96
Codeine	56	77	91	94	99	100	149	149	143	144	137	---
Colchicine	---	s	153	138	123	100	---	s	445	224	140	133
Ephedrine	67	96	100	101	102	100	130	117	135	132	116	112
Epinephrine	---	---	137	116	98	101	---	---	58	66	83	---
Ergotamine	---	174	117	110	110	105	---	291	170	155	145	123
Morphine	130	99	98	98	99	100	138	144	138	139	134	128
Physostigmine	---	---	620	295	130	100	153	132	135	131	130	100
Pilocarpine	198	140	105	104	103	102	126	137	149	133	129	107
Quinidine	s	21	79	82	89	102	s	71	98	124	119	110
Quinine	s	6	34	83	95	97	s	86	105	136	127	122
Strychnine	s	10	13	44	83	103	s	67	78	107	104	117
d-Tubocurarine	---	5	9	34	76	98	---	73	88	93	105	114
Veratrine	---	s	58	109	107	110	---	s	720	510	237	178
Yohimbine	s	116	96	99	95	99	s	110	108	101	110	106

*Each value represents the average of ten separate experiments. The S. E. of the mean for each value was less than ±4 per cent.

†S, Shortening of the muscle occurred during the immersion of the muscle in the solution for five minutes, without addition of acetylcholine or potassium.

DISCUSSION

Shortening of muscle induced by acetylcholine and by potassium involves mechanisms not identical in all details. Acetylcholine and potassium induce different physicochemical changes in the muscle cells[2-6] and even different physical changes in isolated myosin. Acetylcholine sensitivity parallels in many respects the sensitivity of muscle to indirect stimulation, whereas potassium sensitivity often parallels the effect of direct stimulation.[7]

The alkaloids used can be divided into two groups according to their effect on acetylcholine sensitivity: alkaloids increasing the acetylcholine sensitivity of striated muscle and alkaloids with a curarelike effect of decreasing the acetylcholine sensitivity.

The acetylcholine sensitivity of striated muscle was increased by at least two mechanisms: decrease of the activity of cholinesterase[8-17] and direct effect on the striated muscle. That alkaloids exerted a direct effect on the muscle cell may be concluded since changes of acetylcholine sensitivity often did not parallel the degree of inhibition of the activity of cholinesterase,[8-17] and the alkaloids modified the potassium sensitivity of the muscle.

The decreased potassium sensitivity of muscle immersed in epinephrine was, at least partly, due to the fact that epinephrine reduces the potassium content of the muscle cell (as documented by Torda and Wolff[1]). Because d-tubocurarine, in higher concentrations, disintegrates the actomyosin fibers[18] and also decreases the activity of adenosinetriphosphatase,[19] it is likely that the decreased potassium sensitivity of the muscle in the presence of d-tubocurarine was, at least partly, due to these mechanisms.

Curare, atropine, quinine, strychnine, and veratrine (the latter in high concentrations) were shown to have a curarelike effect, that is, these alkaloids abolish the effect of indirect stimulation on striated muscle in concentrations that do not abolish the effect of direct stimulation.[20, 21] d-Tubocurarine, atropine, amphetamine, codeine, cinchonine, quinine, quinidine, strychnine, and veratrine had a curarelike effect (as shown in the present experiments) in that they decreased the acetylcholine sensitivity of the rectus abdominis muscle in relatively low concentrations but did not decrease the potassium sensitivity in the same concentrations.

SUMMARY

1. The effect of eighteen alkaloids on the acetylcholine and potassium sensitivity of striated muscle were investigated.

2. The acetylcholine sensitivity was increased by physostigmine, colchicine, epinephrine, ergotamine, pilocarpine, morphine, and yohimbine (the latter three in higher concentrations), and was decreased by the other alkaloids tested.

3. The potassium sensitivity was increased in the presence of all alkaloids tested (except epinephrine and cocaine), and was decreased by epinephrine and cocaine, and, in higher concentrations, by d-tubocurarine, strychnine, quinine, quinidine, and atropine.

4. d-Tubocurarine, codeine, atropine, amphetamine, cinchonine, quinidine, quinine, strychnine, and veratrine showed a curarelike effect in that they induced a decrease of acetylcholine sensitivity in low concentrations and such concentrations did not modify the potassium sensitivity of the muscle.

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LABORATORY METHODS

DILUTION TECHNIQUE FOR RAPID ESTIMATION OF DIHYDROSTREPTOMYCIN AND OTHER ANTIBACTERIAL AGENTS IN BODY FLUIDS

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THE technique to be described is a simple dilution procedure for the rapid estimation of dihydrostreptomycin,*[1] streptomycin, penicillin, or other antibacterial agents in a fluid medium. Theoretically, dihydrostreptomycin should be readily assayed by many of the rapid methods used for the determination of streptomycin, but some chemical methods[2-4] are no longer applicable due to modification[1, 5] of the structure. The microbiologic, nephelometric methods[1, 6, 7] are generally impossible to apply to blood, and agar plates[8-10] are laborious and cumbersome when used for infrequent samples. A simple dilution method for rapid estimation of antibiotics in the blood is a clinical and research necessity. Such a technique was not found in the literature.

The following procedure, which has been used successfully for over two years for the determination of streptomycin,[11] is based on the profuse growth of a large inoculum of a sensitive test organism and on its ability to reduce a solution of methylene blue added after growth has begun. The reaction produces a clear, easily read end point. The tubes in which growth has occurred are easily distinguished from those in which it has been inhibited by the antibacterial agent; in the latter, the blue color remains unchanged. While the end point can be determined in three hours and the concentration of the antibiotic can be estimated then, a sharper end point is obtained after four hours.

METHOD

The following method is used for the determination of an unknown concentration of dihydrostreptomycin (or streptomycin) in a blood sample, whether whole blood, plasma, or serum. The test organism employed is a strain of *Bacillus prodigiosus (Serratia marcescens)* which reduces methylene blue in the presence of 0.3 unit, but not 0.4 unit, of dihydrostreptomycin per cubic centimeter of 1 per cent peptone in physiologic saline solution. Streptomycin in a concentration of 0.4 μg also inhibits reduction, while a concentration of 0.3 μg per cubic centimeter is not inhibitory.

Procedure (Blood, Plasma, or Serum).—To the first tube of a series of ten sterile small test tubes (13 by 100 mm. or 13 by 150 mm.), each containing 1.0 c.c. of physiologic saline

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solution, is added 1.0 c.c. of the blood (citrated or heparinated) to be assayed for dihydrostreptomycin. With a fresh pipette the blood and saline solution are mixed in the first tube and 1.0 c.c. is transferred to the second tube; another fresh pipette is used to mix and transfer 1.0 c.c. to the third tube, and so on; 1.0 c.c. is discarded from the tenth tube, the last of the series.

A control series is set up in an identical manner, using instead of the unknown blood sample defibrinated sheep or rabbit blood or, preferably, horse serum containing 100 units of dihydrostreptomycin per cubic centimeter. To each of the ten tubes in both the unknown and the control series is added 1.0 c.c. of 10^{-2} dilution of a heavy (sixteen to twenty-four hours' growth at 37° C.) culture of *B. prodigiosus* in physiologic saline solution containing 1.0 per cent peptone. Shake the tubes and incubate at 37° C. for two hours. Then add to each tube 0.2 c.c. of a 1:10,000 solution of methylene blue dissolved in physiologic saline solution. After shaking to obtain an evenly colored mixture, each tube is layered with 0.5 c.c. of mineral oil (liquid petrolatum, U. S. P.). The tubes are then incubated again at 37° C. for another two hours.

To estimate the concentration of dihydrostreptomycin in the unknown blood sample, the number of tubes in the unknown series in which the blue color is unchanged is compared with the number in the control series. Thus, if six tubes in the control series remained blue and the unknown series showed five blue tubes, the latter would contain 50 units; if six tubes in the unknown remained blue, it would contain 100 units, or equal the control; seven blue tubes in the unknown series would indicate a concentration of 200 units, and so forth.

Procedure (Urine or Aqueous Fecal Extract).—The same procedure is used for urine and fecal extracts containing antibiotics. Sixteen to twenty-four hour cultures of *Escherichia coli*, Waksman and associates[11, 12] grown at 37° C. in 1 per cent peptone, have been used in 10^{-3} dilutions for the determination of dihydrostreptomycin or streptomycin in such samples. This coliform organism does not produce reduction when dihydrostreptomycin is present in 1.1 units per cubic centimeter or streptomycin in 1.0 μg per cubic centimeter, but is effective in 1.0 unit of dihydrostreptomycin or 0.9 μg of streptomycin per cubic centimeter. However, a 10^{-2} dilution of *B. prodigiosus*, which grows more slowly than *Esch. coli*, is preferable.

The high levels of streptomycin present in urinary or fecal samples taken during therapy necessitate a tenfold dilution of the original sample, and the final value for the unknown sample is obtained by multiplying the estimate by ten.

DISCUSSION

Sterile glassware is used entirely, but plugs for the test tubes are not necessary. The methylene blue solution and the mineral oil are not sterile. The test may be read one hour after the addition of methylene blue solution and mineral oil, or at any time thereafter. However, reading two to four hours following the second period of incubation is recommended. The number of tubes reduced varies directly with the time of incubation. A late reading not only gives lower sensitivity, but may also render the test inaccurate by allowing contaminants to grow. The latter has not yet been encountered in this laboratory, where no special precautions are observed, and readings at the end of twenty-four hours have been found to be in good agreement with those made earlier, except when the antibiotic was present in low concentration.

In the presence of a 10 per cent or more concentration of blood, for example, in the first and second tubes of the blood dilution series, reduction of methylene blue is not a reliable index of growth manifestations, and nonreduced methylene blue does not necessarily indicate inhibition. The cause of this anomaly is unknown. It is necessary at present to avoid testing whole

blood samples which will give end point results in the initial tubes. Serum and plasma do not react in this way. The testing of serum in place of blood has still other advantages: blood may conveniently be allowed to clot, and serial dilutions may be made without changing pipettes from tube to tube. Under the conditions described, the varying amounts of serum in successive tubes have no noticeable influence on the growth of *B. prodigiosus*.

While sterilization of fresh urine samples is not absolutely necessary, it is recommended that 5 c.c. be heated for twenty to thirty minutes in boiling water before use in antibiotic determination. Dihydrostreptomycin solutions may be sterilized by steam autoclaving for thirty minutes at 115° C. and 15 to 20 pounds of pressure without loss of potency. Solutions of streptomycin, penicillin, or tyrothricin should not be subjected to either treatment. Dilutions of fecal extracts may be tested by the same procedure used for urine.

APPLICATION

These procedures, which are capable of wide application, can be used with any sensitive organism which grows rapidly under the described conditions. In this laboratory, the aforementioned organisms and *Bacillus subtilis, Salmonella typhimurium, Staphylococcus aureus SM, Staphylococcus aureus 209P, and Streptococcus haemolyticus N.Y.5* have been successfully used in the assay of penicillins, streptothricin, tyrothricin, alkyl quaternary ammonium salts, and some aromatic amidines.

Test data are given in Table I. Values obtained by other methods are included[7, 8, 13-15] for purposes of comparison.

TABLE I

SAMPLE	TEST ORGANISM DILUTION	MEDIUM	TIME (HR.)	ORGANISM INHIBITED (CONC./C.C.)	SAMPLE (CONC./C.C.) (CA.)	KNOWN (CONC./C.C.)	OTHER METHODS (CONC./C.C.)
Dihydrostreptomycin in:							
Horse serum	E.c. 10^{-3}	A	4	1.6 units	200 units	100 units	120 units
Fecal extract	S.t. 10^{-3}	A	4	0.8 unit	1,000 units	1,000 units	----
Blood	B.p. 10^{-2}	A	4	0.8 unit	100 units	100 units	----
Urine	B.p. 10^{-2}	A	4	0.8 unit	1,000 units	1,000 units	1,180 units
Streptomycin in:							
Urine	E.c. 10^{-3}	A	4	0.8 μg	4,000 μg	----	3,800 μg
Serum	B.p. 10^{-2}	A	4	0.2 μg	100 μg	100 μg	90 μg
Fecal extract	B.p. 10^{-2}	A	4	0.4 μg	5,000 μg	----	----
Penicillin in:							
Serum	S.a. 10^{-2}	B	3	0.080 unit	1.25 units	----	1.5 units
Blood	S.a. 10^{-3}	B	6	0.030 unit	12.50 units	25 units	----
Physio. saline	S.a. 10^{-3}	B	6	0.010 unit	50.00 units	50 units	43.5 units
Serum	S.h. 10^{-2}	C	6	0.005 unit	1.25 units	----	1.5 units
Quaternary ammonium salt in:							
Urine	E.c. 10^{-3}	A	4	16.00 μg	2 mg.	2 mg.	2.2 mg.
Water	S.t. 10^{-3}	A	4	0.02 μg	10 mg.	10 mg.	9.8 mg.
Tyrothricin in:							
Water	B.s. 10^{-2}	B	3	1 μg	0.25 mg.	0.25 mg.	----
Streptothricin in:							
Water	P.p. 10^{-2}	B	10	16 units	500 units	----	----

E.c., *Esch. coli*; S.t., *S. typhimurium*; B.p., *prodigiosus*; S.a., *Staph. aureus*; B.s., *B. subtilis*; S.h., *Str. haemolyticus*; P.p., *P. pestis*.

A, 1 per cent peptone; B, hormone broth;[16, 17] C, B with 5 per cent serum.

Mg. (milligram) = 1000 μg (microgram).

SUMMARY

A simple microbiologic serial dilution technique has been presented in detail for the four-hour estimation of dihydrostreptomycin or streptomycin in whole blood, plasma, serum, urine, or fecal extractions.

The procedure has also been applied to other antibacterial agents, such as penicillins, streptothricin, tyrothricin, quaternary ammonium salts, and aromatic amidines.

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ESTIMATION OF URIC ACID IN SERUM AND WHOLE BLOOD BY AN ELECTROPHOTOMETRIC MODIFICATION OF FOLIN'S METHOD

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THE purpose of this communication is to report a study of the estimation of uric acid in serum and in whole blood by an electrophotometric modification of Folin's direct macromethod.[1-3] Details of the adaptation of Folin's technique to the electrophotometer, recoveries of uric acid from serum and whole blood, and estimations of the uric acid content of serum and whole blood of normal subjects are presented.

This investigation was stimulated by a report by Jacobson[4] of the satisfactory results of the application to serum of Folin's direct method of estimation of blood uric acid.[1] In thirty experiments, in which uric acid was added to serum (or plasma), the average recovery was 90 per cent and individual recoveries were 80 per cent or higher in all but one instance. The upper limit of the normal range of serum uric acid concentration was 6.0 mg. per cent, and values above this were found in 174 of 177 tests on twenty-one patients with gout. These recoveries and the demonstration of this high incidence of hyperuricemia in gout are in contrast with the findings of many investigators. Thus, in 1941, Bulger and Johns[5] stated that the uncertainty regarding the reliability of methods of estimation of blood uric acid was so great that some laboratories no longer attempted these tests. We were therefore prompted to study Folin's method with special reference to its application to serum and, since whole blood is commonly used for estimation of uric acid, to compare results obtained with serum and whole blood.*

Electrophotometric Modification of Folin's Direct Macromethod.—Preliminary experiments with Folin's technique showed that the color of the blank was not constant. At times it was pale to moderately deep yellow; at other times, although pale yellow at first, it rapidly became pale green. These variations appeared to be due to impurities in the sodium cyanide used in the urea-cyanide reagent† and, to a less extent, to very small changes in the proportions of sodium tungstate and phosphoric acid in the uric acid reagent.[3] A change in the blank occurred whenever a new lot of sodium cyanide was used and sometimes when a new uric acid reagent was prepared. It was therefore decided to adapt the technique to the electrophotometer, since this would permit a correction for the blank with each estimation.

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*In all tests with whole blood the anticoagulant was sodium oxalate and red cells were laked before precipitation of proteins.[6] Unlaked blood filtrates[7] were not used since they yield values which are influenced by a variable degree of extraction of chromogenic material from the red cells.[4]

†Folin[3] recommended the use of Merck's reagent sodium cyanide. In our experience this does not ensure constancy of the blank.

A Fisher electrophotometer with two photoelectric cells, null adjustment, microabsorption cells an a filter of maximum transmission at 525 mμ was used.

Experiments with aqueous solutions of uric acid showed concentration to be directly proportional to the negative logarithm of the transmission factor with amounts of uric acid below 0.6 mg. per cent only (equivalent to a serum or blood uric acid of 6 mg. per cent). The rate of color development was variable and appeared to be markedly influenced by impurities in the sodium cyanide used in the urea-cyanide reagent. With some lots of cyanide color development was slow and a deep blue color was not formed until thirty to forty minutes after addition of uric acid reagent. With other lots color development was more rapid, the blue color was deep at the end of twenty minutes and was replaced by a green color between thirty and forty minutes. A calibration curve could not be used and a standard, equivalent to a blood uric acid of 4 mg. per cent, was set up with each unknown as described by Folin.[1, 3]

Experiments with Folin-Wu filtrates representing 1 in 10 dilutions of serum and blood[3, 6] showed their behavior to be similar to that of aqueous solutions. Rate of color development was variable but paralleled that of the standard set up at the same time with the same reagents.

On the basis of these findings, Folin's direct macromethod was adapted to the electrophotometer as follows. A standard, equivalent to a blood uric acid of 4 mg. per cent, and a blank were set up with each unknown. Readings were made at any time between twenty and forty minutes after addition of uric acid reagent. With the blank in position in the electrophotometer, the galvanometer was adjusted to read zero on the logarithmic scale. Readings of the standard and the unknown were then made. Since the colors of the blank, the standard, and the unknown were not stable, readings were made in as rapid succession as possible. The concentration of uric acid in the unknown was calculated from the readings of the logarithmic scale of the galvanometer. If the value obtained was higher than 6 mg. per cent, the test was repeated with a filtrate diluted with water to obtain a final reading in the range of direct proportionality between concentration and logarithmic scale readings.

The modifications introduced in adapting the technique to the electrophotometer only have been described; otherwise the preparation of reagents and procedure of the test were as described by Folin.[1-3]

Recovery of Uric Acid From Serum and Whole Blood.—Uric acid was added to serum and whole blood in concentrations ranging between 2 and 10 mg. per cent. Filtrates, representing dilutions of serum or blood of 1 in 10, were prepared by the Folin-Wu procedure[3, 6] and estimations before and after addition of uric acid were made by the electrophotometric modification of Folin's direct macromethod. The results (Table I) show that recoveries from whole blood were lower and more variable than those from serum. Demonstration of the inaccuracies involved in analysis of the uric acid content of whole blood is not new and the factors responsible for poor recoveries have been discussed

in the literature.[4, 7, 8] The figures reported here are of interest in providing a comparison between recoveries from serum and whole blood by the same technique.

TABLE I. RECOVERIES OF URIC ACID FROM SERUM AND WHOLE BLOOD

	TOTAL NUMBER OF EXPERIMENTS	RECOVERY OF ADDED URIC ACID*			NUMBER OF EXPERIMENTS WITH RECOVERIES OF 85% OR MORE
		MAXIMUM	MINIMUM	AVERAGE	
Serum	30	110%	78%	94%	29
Whole blood	30	105%	55%	83%	15

*Recoveries were calculated by the formula $\frac{U_2 - U_1}{a} \times 100\%$, in which U_1 and U_2 were amounts of uric acid found before and after addition of uric acid, respectively, and a was the amount added. In some publications recovery is calculated as $\frac{U_2}{U_1 + a} \times 100\%$. The latter method yields higher figures; the discrepancy is especially marked when U_1 is high and a is low.

Variations in the color of the blank and in the rates of color development by standards and unknowns had no influence on recoveries. It should be noted here that these recovery experiments were not done with only one set of reagents. Six lots of sodium cyanide, representing products of three manufacturers, and four preparations of uric acid reagent were used.

These results indicate that serum is to be preferred to whole blood for estimation of uric acid by Folin's method.

Normal Values of Uric Acid Content of Serum and Whole Blood.—Blood was collected from 100 normal individuals with no family history of gout. There were 63 men and 37 women. The maximum, minimum, and average ages were 58, 15, and 29 years respectively. Samples were obtained in the morning one to three hours after breakfast and were not collected under oil.[4] The latter precaution was omitted since it is inconvenient in routine work and we wished to determine normal values with samples collected under the usual conditions of exposure to air. Uric acid in Folin-Wu filtrates of serum and whole blood was estimated by the electrophotometric modification of Folin's direct macromethod.

The results are shown in Tables II and III. As expected from the recoveries, average values obtained with whole blood were lower than those obtained with serum.* Individual values showed considerable variation. Thus, whole blood values were equal to or higher than serum values in 15 of the 100 tests

*The ratios of the difference between the means to the probable error of the difference show the differences between whole blood and serum values to be statistically significant. Statistical formulas used:

$$\sigma \text{ sample} = \sqrt{\frac{\Sigma v^2}{n}}$$

$$\text{P. E. sample} = 0.6745 \times \sigma \text{ sample}$$

$$\sigma \text{ mean} = \frac{\sigma \text{ sample}}{\sqrt{n}}$$

$$\text{P. E. mean} = 0.6745 \times \sigma \text{ mean}$$

$$\sigma(M_1 - M_2) = \sqrt{(\sigma M_1)^2 + (\sigma M_2)^2}$$

$$\text{P. E. } (M_1 - M_2) = 0.6745 \times \sigma(M_1 - M_2)$$

In these formulas: σ, standard deviation; v, individual deviation from mean; n, number of measurements; P. E., probable error; M_1 and M_2, means of two series of measurements.

and, among the remaining 85, differences between serum and whole blood values varied between 0.1 and 2.9 mg. per cent. In individual cases, the uric acid content of serum cannot be predicted from the value obtained using whole blood.

TABLE II. SERUM URIC ACID IN ONE HUNDRED NORMAL SUBJECTS

	NUMBER OF TESTS	SERUM URIC ACID (MG. PER CENT)				
		MAXIMUM	MINIMUM	AVERAGE	PROBABLE ERROR SAMPLE	PROBABLE ERROR MEAN
All cases	100	6.9	2.9	4.5	±0.6	±0.06
Men	63	6.9	2.9	4.8	±0.6	±0.08
Women	37	5.7	2.9	4.0	±0.4	±0.07

Our normal values of serum uric acid are slightly higher than those reported by Jacobson.[4] This is probably explained by the fact that our samples of blood were not protected from exposure to air, whereas Jacobson collected blood under oil. This investigator has shown that exposure of blood to air may increase the apparent uric acid content of serum by as much as 0.6 mg. per cent. It should also be noted that Jacobson's tests were done while the subject was in the fasting state, whereas those reported here were done one to three hours after breakfast. However, there was no evidence that our highest values were related to consumption of breakfasts of high purine contents.

TABLE III. WHOLE BLOOD URIC ACID IN ONE HUNDRED NORMAL SUBJECTS

	NUMBER OF TESTS	WHOLE BLOOD URIC ACID (MG. PER CENT)				
		MAXIMUM	MINIMUM	AVERAGE	PROBABLE ERROR SAMPLE	PROBABLE ERROR MEAN
All cases	100	6.4	2.2	3.8	±0.6	±0.06
Men	63	6.4	2.5	4.1	±0.6	±0.08
Women	37	4.4	2.2	3.2	±0.4	±0.07

Nothing would be gained from a detailed comparison of our data with those obtained by other techniques[5, 9-13] since values depend to a considerable extent on the technique employed. In general, values obtained with serum or plasma are higher than those obtained with whole blood and indicate the upper limit of the normal range for serum or plasma to be 6 to 7 mg. per cent.[14]

As shown in Tables II and III our data agree with those reported elsewhere[5, 10, 12] in demonstrating lower values in women than in men.*

SUMMARY

1. An electrophotometric modification of Folin's direct macromethod of estimation of uric acid in blood is described. This was developed to eliminate errors due to variable blanks met with in this technique.

2. With this method recoveries of uric acid from serum were higher and less variable than those from whole blood. Serum is therefore to be preferred to whole blood for estimation of uric acid by this technique.

*The ratios of the difference between the means to the probable error of the difference show these differences between men and women to be statistically significant.

3. Estimations of the uric acid content of whole blood and serum were made in 100 normal individuals with no family history of gout. The average values obtained with whole blood were significantly lower than those obtained with serum, but individual values showed considerable variation. In individual cases the uric acid content of serum cannot be predicted from analysis of whole blood.

The normal range of concentration of uric acid in serum was 2.9 to 6.9 mg. per cent.

Whole blood and serum uric acid concentrations were significantly lower in women than in men.

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A DERMAL PLETHYSMOGRAPH FOR THE RECORDING OF SKIN-WHEALING REACTIONS

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PREVIOUS comparative studies on intradermal wheals, initiated shortly after the introduction of the McClure-Aldrich saline wheal disappearance tests,[1, 2] were disappointing because of lack of quantitative methods for the measurement of wheal volumes. The first effort at quantitation was probably that of Schade[3] in 1912 who used a weighted disk placed on top of saline wheals and recorded kymographically the slow sinking and disappearance of these wheals. Alexander and co-workers[4-6] traced the irregular wheal boundary with a planimeter and used the recording as an index of wheal size. Gowen[7] suggested a practical clinical method of approximating the size of wheals by the use of a thin 4 cm. square glass with three circles, 1 cm., 2 cm., and 3 cm. in diameter, marked on its surface. For measurements, the glass square was placed over the wheal with the point of injection in the center of the 1 cm. circle. A rubber stamp with circles of the same size was used to record and transfer a rough outline of the wheal size to the patient's chart.

Intradermal wheals have been used in animals and man to compare the degree of irritation caused by local anesthetics.[8] An accurate means of wheal measurement would suggest extending this simple test to study the intradermal tissue tolerance of other new drugs. The tendency of many drugs to cause an inflammatory reaction or cellular irritation, as well as the characteristic curve of absorption, might thus be determined by the use of various concentrations of the test substance. The results of intradermal injections could then be correlated with the intramuscular and subcutaneous reactions to the drug. During the course of studies on new local anesthetics a distinct need was felt for a more accurate means of measuring wheals and cutaneous reaction to intradermal injections. Accordingly the following dermal plethysmograph was designed.

APPARATUS

The dermal plethysmograph (Fig. 1) consists of a 20 to 30 cm. glass capillary tube (bore, approximately 1 mm.) opening into a hollowed-out rubber base. Using rubber cement, a thin rubber membrane is cemented to the rubber base of the plethysmograph and the edges of the membrane are then trimmed with scissors. When the tube and rubber base are filled with an 80 per cent alcoholic solution of toluidine or methylene blue, any pressure against the rubber membrane causes the blue solution to rise in the tube.

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When weighted with sufficient lead to make the entire instrument weigh 100 grams it is extremely sensitive to volumetric changes of the skin surface. The pulse is seen as a regular rise and fall of the blue column as the instrument rests on the subject's arm. Greater weight may cause a gradual depression of the supporting skin and a resultant rise of the blue column of fluid over a forty-five minute period. With an external diameter of 37 mm. and an internal opening of 21 mm., the instrument applies 100 grams of weight over

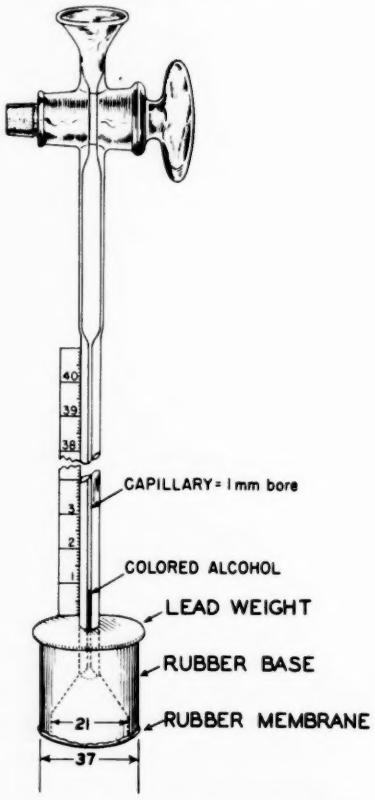

Fig. 1.—Dermal plethysmograph.

an area of 728 sq. mm., or approximately 50 grams per 350 square millimeters. In this regard Schade[3] found that 50 grams per 50 sq. mm. did not exceed the elastic limits of the skin when applied for a two-minute period. Control readings indicate that 100 grams per 728 sq. mm. does not exceed the elastic limits when applied for a forty-five minute period, since no spontaneous rise occurs during this time. That changes in skin temperature are not important in the readings of the skin plethysmograph is shown by Fig. 2. The bases of three plethysmographs were immersed to a depth of 2 mm. in a water bath and the temperature was raised slowly from 22 (room temperature) to 37.5° C. Over the temperature range encountered in the skin of the forearm (33 to 37° C.) the readings of the plethysmographs increased only 1 to 2 mm., which

is not sufficient to necessitate a correction. When the capillary is calibrated with mercury it is found that a capillary of approximately 1.0 mm. bore will contain 0.009 c.cm. of water per centimeter length. Thus with individually calibrated capillaries, graph paper may be used to measure the changes in volume of the skin surface. Knowing that the wheal is made in the dermis,[9, 10]

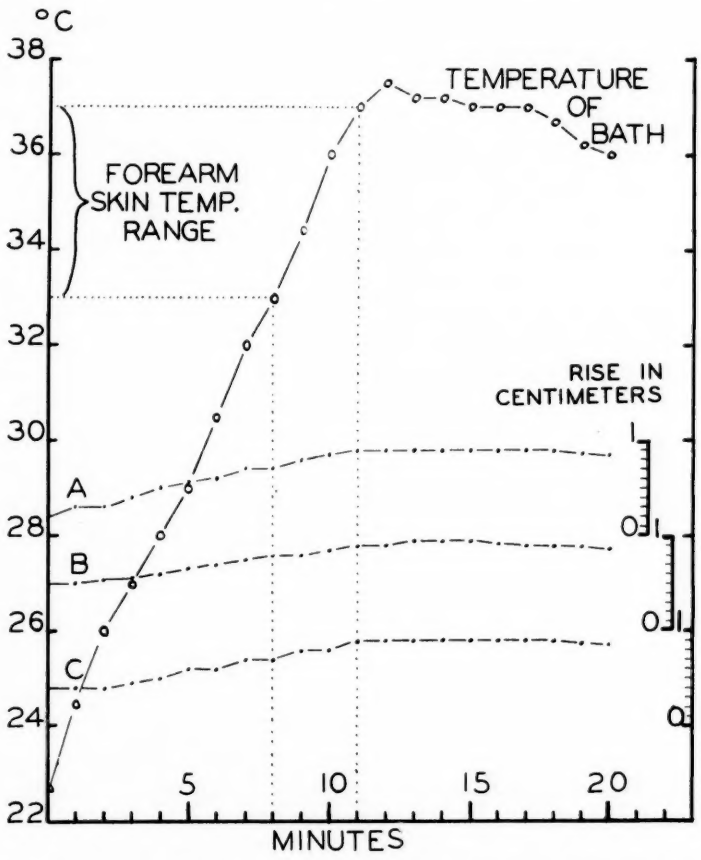

Fig. 2.—The possible effect of skin temperature on the volumetric readings of the dermal plethysmograph. *A*, *B*, *C*, readings in centimeters of three dermal plethysmographs.

one can assume that at least one-half is submerged in the soft subcutaneous tissues so that only the top half of any given wheal could possibly be measured. The instrument is calculated on this basis to be 63 per cent effective. That is, when 0.2 c.c. wheals were made with serum, the total volume measured is 0.063 c.c. rather than the theoretic value of 0.10 cubic centimeter.

PROCEDURE

Most consistent results are obtained with a trained and relaxed subject reclining on a bed with the arm immobilized with small sand bags. A series of control readings are taken on a level area of the flexor surface of the subject's forearm by lifting and replacing the plethysmograph several times. The

average of these control readings is taken as a base reading. Each time before removing the plethysmograph from the skin the stopcock should be closed to prevent bulging of the rubber membrane due to pressure from the column of fluid. It is opened upon replacing. After the control readings have reached a constant value, the instrument is removed from the arm and a wheal is raised in the exact center of the area where the dermal plethysmograph had been resting. The wheal is made by injecting intradermally 0.2 c.c. of the sterile test solution using a ¼ c.c. tuberculin syringe and a 27-gauge one-fourth inch needle. The stop watch is started after the needle is inserted and immediately before the solution is injected. The dermal plethysmograph is replaced over the identical area, and readings are taken at thirty and sixty seconds and at every minute thereafter for a period of forty-five minutes.

With the enormous pressures required for whealing,[11] some variation in wheal size may result from leakage of a test solution either back around the syringe plunger in a poorly fitting syringe or between the syringe and the needle. To avoid this in quantitative work, each syringe must be carefully tested for leaks by the following procedure: a needle is firmly attached to the syringe, then inserted into a solid rubber stopper; if pressure exerted on the syringe causes the fluid in the syringe to escape at the bevel or back along the piston, the syringe is discarded. Puncturing of the basal layer of the corium (as by poor technique) also causes a smaller and more rapidly disappearing wheal. (Some evidence has been obtained which indicates that a markedly irritating solution may regularly result in smaller wheals.) Finally, the primary factors in the size and disappearance of wheals are the varying abilities of the solutions to spread between the cell walls, to penetrate the tissues and the cell walls, to cause cellular swelling, to increase capillary permeability, and to cause delayed inflammatory reactions.

Since the human subject must lie supine for forty-five minutes we have found it expedient to use three dermal plethysmographs simultaneously and make three wheals in rapid succession in the skin of the subject's forearm. The tabulation of data from three plethysmographs at minute intervals is easily within the skill of a trained operator.

RESULTS

Example I. Reaction to Insect Toxin.—Subject W. F., a 21-year-old man known to have an immediate whealing reaction to mosquito bites, was chosen for this experiment. A single mosquito *(Aedes aegypti)* was allowed to bite the subject in the volar surface of the forearm. After the mosquito had become engorged and had withdrawn his proboscis, the dermal plethysmograph was placed on the site and development of the wheal measured (Fig. 3, *A*). The experiment, when repeated the next day, produced a similar type of whealing reaction (Fig. 3, *B*). The response curves are sufficiently analogous to indicate the usefulness of this method of recording when immediate reactions to an injected substance are anticipated.

Example II. Reaction to Histamine Diphosphate.—Two subjects were chosen for this experiment. Subject A, a 20-year-old man, was known to

react quite markedly to intradermal histamine, while Subject B, a 38-year-old man, was known to have a less marked response. Neither subject had an allergic history. The skin of the subject was cleaned with 70 per cent alcohol, and a drop of histamine diphosphate, 1:1,000, was placed on the clean dry skin. A sterile 27-gauge one-fourth inch needle was then used to make two punctures through the dermis beneath the drop of histamine. The excess histamine was blotted off with gauze and the recording instrument was placed over the site. This technique results in a uniform whealing response when used in

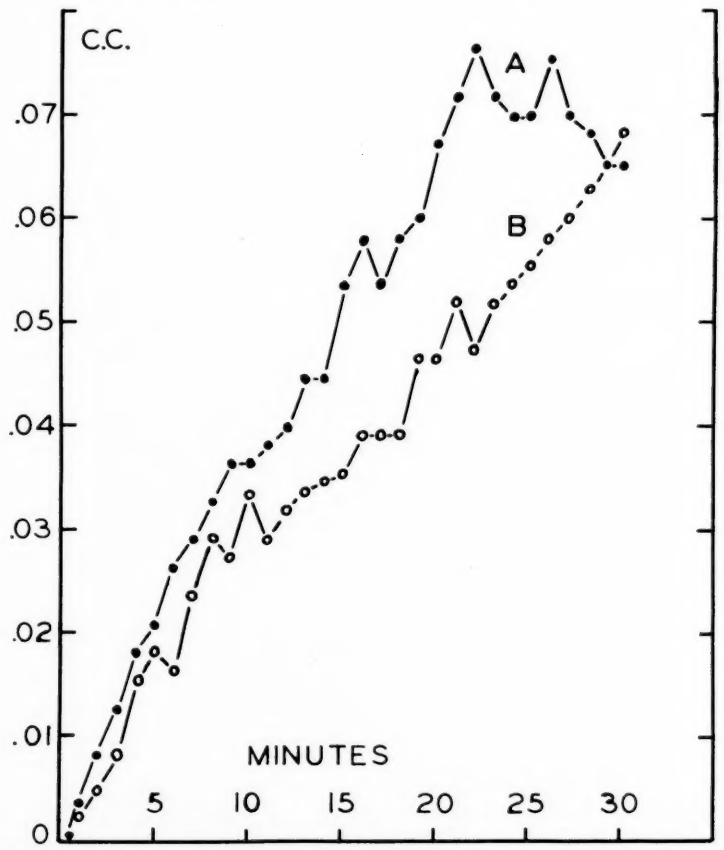

Fig. 3.—The response of a normal subject to mosquito bites (*Aedes Egypti*) on two successive days.

experimental subjects during the summer months. (In winter months we have found the skin to be much less responsive.) It is evident from inspection of the curves obtained on three successive days (Fig. 4) in each subject that Subject A has a consistently greater response to the whealing action of histamine than Subject B who has a consistently minimal response. These separate families of curves are distinct and probably reflect the sensitivity of these two subjects when histamine is applied in this manner.

Example III. Disappearance Curves of Saline and Glucose.—Previous examples of the application of the dermal plethysmograph have shown charac-

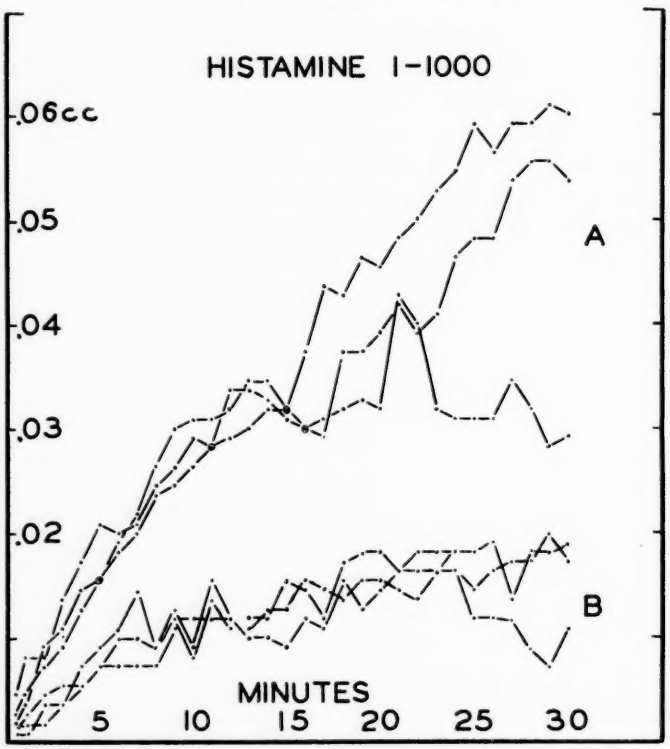

Fig. 4.—The response of two normal subjects, *A* and *B*, to histamine on three successive days.

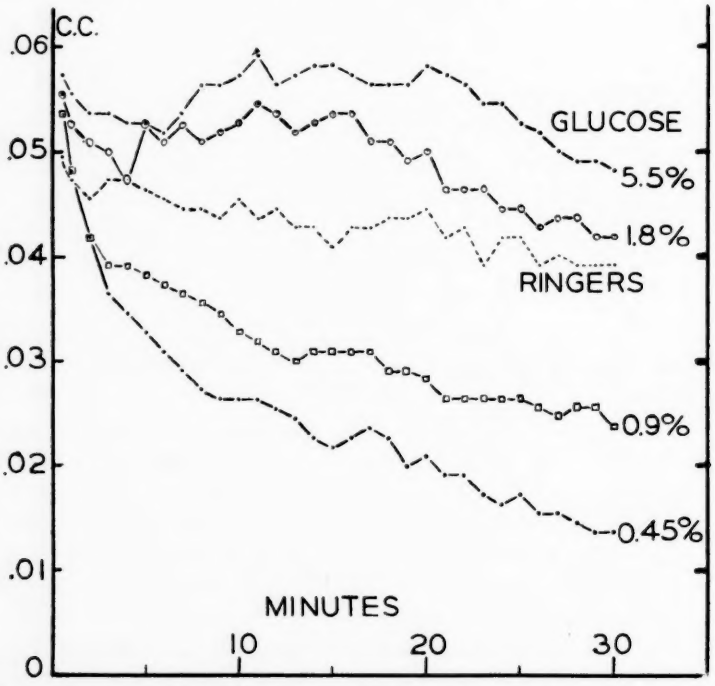

Fig. 5.—Wheal disappearance times (average of five subjects) when solutions of varying tonicity are injected intradermally.

teristic curves of wheal growth. Wheals of various substances in solutions (such as local anesthetics, irritant drugs, and saline) can be made, the wheal size measured, and a characteristic wheal disappearance curve obtained for each substance. The average disappearance curve of five subjects injected with various saline solutions and glucose is characteristic and predictable (Fig. 5). Clinically we know that 5.5 per cent glucose solution is more irritating than 0.9 per cent NaCl solution. The rising plateau curve obtained with glucose simulates the curves of other irritant substances such as nupercaine 1:500 or diothane 1:500.* Sodium chloride, 0.45 per cent, most closely approximates the saline level of the extracellular fluid and in these experiments disappears most rapidly from an intradermal site. Sodium chloride, 1.8 per cent, has an irritant quality similar to that of glucose.

SUMMARY

A dermal plethysmograph (whealometer) has been constructed which measures with a known degree of accuracy the growth and disappearance rate of dermal wheals. Wheal measurement is suggested as a useful procedure for the study of histamine response or other immediate whealing responses and for the determination of the rate of disappearance of various substances injected intradermally.

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*To be reported in a paper on local anesthetics.

PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twentieth Annual Meeting
Chicago, Ill., Oct. 31 and Nov. 1, 1947

PROGRAM

SCIENTIFIC PROGRAM—OCTOBER 31, 1947

FRIDAY MORNING, 9:15 A.M.

1. EFFECT OF URETHANE ON LEUCEMIA

PAUL L. BEDINGER, M.D. (BY INVITATION), HENRY G. PONCHER, M.D., AND LOUIS R. LIMARZI, M.D., CHICAGO, ILL.

Recent investigation has shown that urethane (ethyl carbamate) produces a marked drop in the leucocyte count of both human leucemia and mouse myelogenous leucemia. Normal leucocytes appear to be more refractory to urethane than do leucemic cells. In human leucemia following the oral administration of urethane over a period of several months a favorable effect has been reported in the subjective symptoms, lymphadenopathy, hepatosplenomegaly, and the leucocyte blood pattern. The exact action of urethane is not understood, but a mechanism of inhibition of mitosis of immature cells has been suggested.

Seventeen children and adults with acute leucemia, fourteen patients with chronic lymphatic leucemia, four with chronic myeloid leucemia, and one with monocytic leucemia (Schilling type) were treated with urethane. The drug was given orally in simple syrup or as 7½ grain tablets. Because of intolerance in some cases, mainly shown by nausea and vomiting, 5 grain enteric-coated tablets proved satisfactory. The average daily dose of the drug ranged from 1 to 5 Gm. given over a period of two days to six months.

In the patients with acute leucemia little or no benefit was observed from the use of urethane, although a rapid and marked fall in the leucocyte count was noted in all cases. With the exception of one child who showed an apparent temporary remission in the blood and bone marrow pattern without clinical improvement, all the other patients still presented the blood and bone marrow findings of acute leucemia. The severe anemia and thrombopenia necessitated the use of frequent blood transfusions in spite of the drop in leucocyte count during the administration of urethane. Terminal cases of acute leucemia showed a rise in the leucocyte count in spite of the administration of urethane. Eleven of these patients have died and in several of the cases at autopsy the hemopoietic organs, as well as the others, showed a general infiltration of tissues by leucemic cells.

In chronic myeloid leucemia and chronic lymphatic leucemia urethane produces a gradual or rapid fall in the leucocyte count with apparent symptomatic improvement. Enlarged lymph nodes and enlarged spleen decreased in size, but in no case did they disappear. Although a more mature type of blood pattern was observed in the patients with chronic myeloid leucemia following treatment, immature cells of the myeloid series were always present in the

peripheral blood. In the patients with chronic lymphatic leucemia, the fall in the leucocyte count was associated with an improvement in the blood and bone marrow picture, although in no case was a complete hematologic remission induced by the drug. Improvement in the hemoglobin and erythrocyte count was noted in several cases. There is some indication that remissions induced by roentgen treatment may be prolonged by the use of urethane. No clinical or hematologic improvement was noted in the patient with monocytic leucemia.

Urethane produces varying degrees of toxic granulations in the neutrophilic leucocytes which is especially marked in the cases of acute leucemia. Urethane was without effect on the basal metabolic rate and certain blood chemical constituents including uric acid. The tentative conclusions are that (1) in patients with acute leucemia urethane in oral doses of 1 to 5 Gm. daily will produce moderate to marked fall in the leucocyte count during a period of one to several weeks; (2) in patients with chronic lymphatic leucemia and chronic myeloid leucemia there is a tendency for the blood pattern to approach a more mature type with a decrease in the size of the lymph nodes and spleen; (3) the temporary remission induced by the drug in chronic leucemia is never as complete as that following roentgen therapy; (4) there is no indication that urethane has any permanent effect on the leucemic process.

2. VARIATIONS IN BLOOD PLATELETS IN ALLERGIC INDIVIDUALS FOLLOWING THE INGESTION OF TEST FOODS

FREDERICK J. POHLE, M.D., AND EPHRAIM B. COHEN, M.D. (BY INVITATION)
MADISON, WIS.

Purpura as a manifestation of allergy is well known. Such hemorrhagic disorders are usually nonthrombocytopenic, although in rare instances reference has been made to cases of thrombocytopenic purpura presumably due to a food sensitivity. Squier and Madison, particularly, have emphasized the role of food allergy in the etiology of certain cases of thrombocytopenic purpura. The purpose of the present investigation was to quantitate the platelet response in allergic individuals to the ingestion of test foods.

Twenty sets of observations were made on sixteen allergic subjects. Under basal conditions, leucocyte and platelet counts were performed twice at fifteen-minute intervals prior to ingestion of a standard portion of a test food to which the subject was known to be sensitive. The postprandial blood counts were done at fifteen, thirty, sixty, and ninety minutes. Tourniquet tests were done immediately prior to the feeding and were repeated on the opposite arm sixty minutes later. Platelets were enumerated by one of us (F. J. P.) employing a direct method which, in our hands, admits a technical error of approximately ±4 per cent.

A positive leucopenic index, as evidenced by a postprandial decrease of 10 per cent or more as suggested by Randolph, was demonstrated in seventeen of the twenty studies. On the same basis, a positive "thrombopenic index" was observed fourteen times. The average change in the leucocyte count for the entire group was −22 per cent; the average change in the platelet count was −19 per cent. In fifteen of the twenty sets of observations the subject demonstrated clinical manifestations of sensitivity to the test food. The tourniquet test became positive after the ingestion of the offending food in two individuals. These two patients showed a decrease in platelets of 31 to 33 per cent, respectively. Similar observations of the leucocyte and platelet counts and the tourniquet test were made on a control group of nonallergic individuals, none of whom demonstrated these changes.

These data suggest that this clinical method may aid in the detection of food allergies and that this type of observation may be of value in the study and management of certain thrombopenic states.

3. NEUTRALIZATION OF HEPARIN WITH PROTAMINE (SALMINE)

THOMAS W. PARKIN, M.D. (BY INVITATION), AND WALTER F. KVALE, M.D.
ROCHESTER, MINN.

Hemorrhage due to a prolonged blood coagulation time resulting from the administration of heparin can be controlled only by discontinuing the administration of heparin and by giving blood transfusion. The effects of neither of these procedures may be sufficiently prompt to prevent the hemorrhage from reaching serious proportions.

Certain protamines are known to neutralize heparin in vitro and in animals. Protamines also appear to have certain toxic effects when given to different species of animals. The assumption appears in the literature that on the basis of animal studies protamines cannot be administered safely to human beings. On the contrary, in 1938, Jorpes reported from Sweden that the protamine, clupeine, can be injected intravenously in man to neutralize heparin. In view of the conflicting reports in the literature, further work seemed necessary.

The purpose of this investigation was to determine (1) by work on animals the toxicity of the protamine, salmine, (2) whether salmine can be administered safely to man, and (3) the dosage of salmine required to neutralize the anticoagulant effect of a standard dose of heparin in man.

In vitro studies demonstrated that 1.5 mg. of salmine neutralized 1.0 mg. of heparin, thus showing that there is quantitative neutralization. The lethal dose of salmine in the guinea pig was 6.0 mg. per 100 grams of body weight. Salmine injected intravenously in doses of 90.0 mg. per kilogram in the unanesthetized rabbit and 2.0 mg. per kilogram in the unanesthetized dog produced no reactions. The intravenous injection of salmine in doses of 10.0 mg. per kilogram in the anesthetized rabbit and 2.0 mg. per kilogram in the anesthetized dog produced transient drops in arterial blood pressure. No evidence of anaphylaxis was observed.

Intravenous injections of salmine neutralized heparin which was injected intravenously in dogs. In the dog, elevated coagulation times resulting from the intramuscular injection of heparin in the Pitkin menstruum were temporarily returned to normal levels by single intravenous injections of salmine.

The anticoagulant effect of intravenous injections of heparin in man was studied by determining coagulation times at fifteen- to thirty-minute intervals for a period of three hours. Coagulation times were determined by the Lee-White method with a thermostatically controlled water bath at 37° C. It was found that coagulation times were elevated four times the normal (four minutes) one hour after injection and that then they gradually returned to pre-heparin levels in three hours. With this to serve as a control, 50 mg. of heparin were injected intravenously into ten patients and fifteen minutes later salmine was injected intravenously in doses ranging from 15 to 50 milligrams. In the patients receiving 40 to 50 mg. of salmine, the elevated coagulation times were returned to normal levels within five minutes.

No toxic reactions were noted in any patient during or after the injections of salmine. It is concluded that the intravenous injection of 40 to 50 mg. of salmine promptly neutralizes the anticoagulant effect of 50 mg. of heparin and that such doses of salmine, when given slowly, produce no reactions.

4. SURVIVAL OF TRANSFUSED SICKLE CELLS IN NORMAL SUBJECTS AND OF NORMAL RED BLOOD CELLS IN PATIENTS WITH SICKLE CELL ANEMIA

SHEILA T. E. CALLENDER, M.D.,* AND JAMES F. NICKEL, A.B., ST. LOUIS, MO.
(INTRODUCED BY CARL V. MOORE, M.D.)

This report describes observations made on (1) the survival of normal red blood cells transfused into patients with sickle cell anemia, (2) the survival of red blood cells from patients with sickle cell anemia transfused into normal subjects, and (3) the survival of red blood cells from persons with the sickle cell trait transfused into normal subjects. The Ashby technique of differential agglutination was used. By this method, the time of survival of normal cells in normal recipients has been found to be about 120 days; roughly one-half of the transfused cells disappear within 60 days. Results obtained confirm evidence indicating that the abnormality in sickle cell anemia is an inherent defect in the red blood cells and demonstrate a difference in behavior of erythrocytes from patients with sickle cell anemia and the sickle cell trait.

Normal red blood cells were transfused into three patients with sickle cell anemia. In all three, the survival of the donor cells was similar to that found in normal subjects. This suggests that there is neither a humoral factor in sickle cell anemia nor any element of increased phagocytosis which would accelerate destruction of normal cells.

Four reverse experiments were made using donors with sickle cell anemia and normal recipient subjects. The transfused cells disappeared rapidly from the circulation; nearly all donor erythrocytes were destroyed within thirty days. In one observation, one patient with sickle cell anemia served as a donor and another as the recipient; the transfused cells showed a comparable rapid destruction. A crude measure of the survival of sickle cells in the normal recipients was also made by study of sealed, moist preparations of capillary blood. Sickled donor cells could readily be demonstrated for only about three weeks after transfusion.

Blood from four healthy donors with the sickle cell trait was also given to normal subjects or to patients with mild hypochromic anemia. The survival of these cells was distinctly longer than that from donors with true sickle cell anemia. Four weeks after transfusion, at a time when cells from sickle cell anemia had largely disappeared from the normal recipients' circulation, more than one-half of the sickle cell trait erythrocytes were still circulating. Since these observations are currently in progress, the final survival rates cannot now be given.

The results obtained in this study confirm the evidence that there is an inherent defect in the erythrocytes of patients with sickle cell anemia which is responsible for the accelerated red cell destruction found in that disease. They also demonstrate that cells from subjects with the sickle cell trait have a longer survival time than cells from subjects with sickle cell anemia when transfused into normal recipient subjects. Whether this difference in behavior between sickle cell anemia and sickle cell trait cells is related to the ease with which sickling occurs or to some other qualitative difference in the erythrocytes has not yet been discovered.

*Rockefeller Fellow from the Nuffield Department of Medicine, Oxford, England.

5. EFFECT OF EXTRINSIC FACTOR, LIVER EXTRACT, AND FOLIC ACID ON INDUCED MACROCYTIC ANEMIA OF SWINE

ROBERT W. HEINLE, M.D., ARNOLD D. WELCH, M.D. (BY INVITATION), WALTER L. GEORGE, M.D. (BY INVITATION), MARTIN EPSTEIN, M.D. (BY INVITATION), AND JACK A. PRITCHARD, M.D. (BY INVITATION)
CLEVELAND, OHIO

Production of macrocytic anemia in a pig by administration of a folic acid-deficient diet with added crude folic acid antagonist has been described by Welch, Heinle, and colleagues. Subsequent studies show the anemia to be associated with megaloblastic hyperplasia of the marrow.

In a previous report such an animal was shown to obtain a hematologic as well as very evident clinical response following the administration of a source of extrinsic factor together with normal human gastric juice. The use of the latter was probably unnecessary, as demonstrated by later experiments. These animals do not develop histamine-fast achlorhydria.

Following the described response, the same pig was allowed to relapse and was given a single injection of 15 units of purified liver extract. This was followed by another hematologic and clinical response.

Another pig with induced anemia received four daily intramuscular injections of 10 mg. of folic acid. This was followed by rapid and marked hematologic and clinical improvement.

A third pig developed severe anemia and neutropenia and became critically ill, appearing to be in a moribund state. Administration of two intramuscular injections of 15 units of purified liver extract, 10 mg. of folic acid, and 35 mg. of niacinamide caused rapid and marked hematologic and clinical improvement.

Bethell and co-workers and Welch and co-workers have offered evidence that a substance (s) in purified liver extract may contribute to the proper utilization of conjugates of folic acid by patients with pernicious anemia in relapse. Results obtained in pigs, however, indicate that extrinsic factor and purified liver extracts are effective even though folic acid is not available to the animal.

Folic acid and its conjugates are not in themselves extrinsic factor as evidenced by the facts that folic acid is effective in pernicious anemia without added intrinsic factor and, further, materials free of demonstrable folic acid or its conjugates may be potent sources of extrinsic factor.

It has been demonstrated by several investigators that purified liver extracts effective in pernicious anemia are devoid of significant amounts of folic acid.

In view of recent experimental data, Welch has proposed that folic acid and the antipernicious anemia principle of liver may function via unrelated chemical pathways. While one is reluctant to believe that these substances have no direct or sequential relationship, it can be stated that data available at this time fail to support the idea of such relationship.

6. FURTHER STUDIES OF THE HYPERSPLENIC MECHANISM IN HUMAN DISEASE SYNDROMES

CHARLES A. DOAN, M.D., AND CLAUDE-STARR WRIGHT, M.D.
COLUMBUS, OHIO

Hypersplenic syndromes may present as acute surgical emergencies or as chronic invalidism and may involve the red cells, and/or neutrophiles, and/or thrombocytes. They may reflect pathology inherent and primary in the spleen or secondary to some generalized systemic disease. Splenectomy is the only satisfactory treatment to date.

Diagnosis is dependent upon (1) an adequate history and supporting physical findings (with or without gross splenic enlargement); (2) peripheral cytopenia; (3) sternal marrow aspiration showing normal maturation with compensatory cellular hyperplasia of all deficient circulating elements; (4) an adrenalin test "biopsy" to reveal splenic hypersequestration of all involved cell strains.

The *mechanism* resulting in more or less profound peripheral cytopenia probably reflects both cellular and humoral factors: Parenchymal cellular hypersequestration + excessive concentrations of lysolecithin (or some similar lytic enzyme or enzymes) + R-E cell hyperplasia and hyperphagocytosis = hemolytic anemia, neutropenia, and/or thrombocytopenia. That the hyperplastic bone marrow is actually, as well as apparently, functionally effective in its delivery of essential elements may be seen (1) by immediate direct studies of living marrow cell preparations and (2) by studies at the operating table of splenic artery and vein blood before and after adrenalin induced contraction of this organ. As many as 350,000 platelets have been discovered entering the spleen with only 17,000 leaving it, whereas upon adrenalin introduction via the splenic artery the splenic vein and peripheral capillaries promptly show numbers of platelets leaving the spleen comparable to those carried by the artery. Furthermore, supravital scrapings of the freshly removed spleen show direct microscopic evidence of excessive R-E cell hyperplasia and hyperphagocytosis. In nine pathologic spleens removed for hypersplenism a quantitative increase of lecithinase activity was demonstrated by Scheff and Awny in our laboratory in sharp contrast with two normal control spleens.

Certain clinical and hematologic *recurrences* of hypersplenism may be caused by overlooked accessory splenic tissue, or by its subsequent hypertrophy, or by accidentally implanted "splenosis" fragments. Usually the original hematologic syndrome is repeated, but not necessarily. In one of our series of 216 patients diagnosed on the basis of a hypersplenic mechanism the original problem was that of hemolytic anemia. Splenectomy promptly and permanently alleviated this acute critical danger. Eighteen months later a thrombocytopenic crisis developed and surgical re-exploration, after an appropriate diagnostic study, revealed a 5 gram accessory spleen (extraperitoneal in location at the lower pole of the left kidney), the removal of which completely, and promptly, and permanently restored the platelet equilibrium to normal with cessation of all purpuric manifestations. Recurrences have also been found on surgical re-exploration to be due to a generalized R-E cell hyperplasia and hyperphagocytosis involving liver, lymph nodes, and bone marrow, in which case symptomatic therapy only is available.

The pathologic tissue *classification* recognized in our series of patients covering the past seventeen years, as studied by von Haam and Awny, are:

PRIMARY			
Congenital hemolytic icterus	72	Recurrences	
Thrombocytopenic purpura	62	Accessory spleens	3
Splenic neutropenia	11	Generalized R-E cell hyperphagocytosis	3
Splenic panhematopenia	10		
SECONDARY			
(1) Congestive splenomegaly		(4) Inflammatory splenomegalies	
Banti's syndrome	36	Tuberculosis	1
Felty's syndrome	3	Syphilis	1
Acquired hemolytic icterus	6	Moniliasis	1
(2) Infiltrative splenomegaly		Boeck's sarcoid	1
Gaucher's disease	3	Hodgkin's syndrome	3
Xanthomatosis	1	(5) Neoplastic splenomegaly	
(3) Hemoblastic splenomegaly		Retothelio sarcoma	1
Lymphatic leukemia	1	Hemangioma	2
Myelogenous leukemia	1		

The *hypersplenic mechanism*, as now conceived, is an adequate explanation for a rather wide range of human clinical and hematologic syndromes, hitherto considered to be distinct and sharply separated etiologic entities.

7. THE PRESIDENT'S ADDRESS

8. PROGRESS REPORT ON A STUDY OF VASCULAR CHANGES IN THE SKIN IN PREGNANCY

WILLIAM B. BEAN, M.D., MORRIS W. DEXTER, M.D. (BY INVITATION), ROBERT C. COGSWELL, M.D. (BY INVITATION), AND JAMES EMBICK, M.D. (BY INVITATION), CINCINNATI, OHIO

As a continuation of investigations on vascular spiders and palmar erythema in chronic disease of the liver, we have observed similar phenomena in 1,200 pregnant women, 278 of whom have been followed during pregnancy and for at least six weeks after delivery. In the smaller group observed during and after pregnancy there were 207 Negro women and 71 white women. Vascular spiders have been found in 60 per cent of the white women and only 15 per cent of the Negroes, while palmar erythema was found more frequently (49 per cent) in white than in Negro women (31 per cent). These signs tend to appear during the first two trimesters of pregnancy but may originate during the last months of pregnancy. In a control group of nonpregnant women vascular spiders were found in 16 per cent of the white women and none of the Negro women, while palmar erythema was noted in 5 per cent of the white and 4 per cent of the Negro women.

Both vascular spiders and palmar erythema tend to fade at the time of delivery and in approximately 80 per cent of the cases the lesions can no longer be detected six weeks after delivery. We have found no significant difference in disappearance rate between white and Negro women. Analysis of age, season, parity, sex and viability of child, toxemia, and other factors has revealed no special relation to the incidence of vascular changes in the skin. Liver function tests during and after pregnancy have not been significantly different in those with vascular spiders, palmar erythema, or both, and those without the lesions. Studies of the excretion of steroid hormones is in progress, but data are not yet adequate for evaluation.

9. HEREDITARY HEMORRHAGIC TELANGIECTASIA IN ASSOCIATION WITH CEREBRAL MANIFESTATIONS AND PULMONARY ARTERIOVENOUS ANEURYSM

CHARLES F. WILLIAMS, M.D. (BY INVITATION), ST. PAUL, MINN., AND EDMUND B. FLINK, M.D., MINNEAPOLIS, MINN.

Within the last year two cases of hereditary hemorrhagic telangiectasia or Osler's disease were encountered on the medical service of the Ancker Hospital. Both patients presented findings which satisfy the diagnostic criteria of Osler's disease. Both had a family history of repeated epistaxis in several members and in two generations. Both had small spiderlike angiomas on the face and neck and some punctate angiomas on the skin and mucosa of the mouth and nose which bled with arterial spurting. The first patient was anemic at death undoubtedly from chronic blood loss. The second patient had cyanosis and mild polycythemia because of a pulmonary arteriovenous aneurysm. The first patient, who was mentally deficient, had been observed from the age of 32 years at the Ancker Hospital. He had never had hypertension. At the age of 37 years he had an episode of hemiplegia, after which he continued to have fainting spells and hemiparesis. At the age of 46 years he was hospitalized because of sudden temporary loss of consciousness. Eight days later he became comatose and died in twenty-four hours. No autopsy was obtained, but it is probable from clinical evidence that he had suffered an intracerebral or subarachnoid hemorrhage. The second patient had also been admitted to the Ancker Hospital at the age of 30 years because of convulsions and unconsciousness. A diagnosis of idiopathic epilepsy was made. He had no more convulsions until the age of 33, at which time he was hospitalized because of a scalp injury sustained during a convulsion. On auscultation of the chest one could hear a systolic murmur in the second left interspace anteriorly. The murmur was accentuated by inspiration. Fluoroscopy revealed a pulsating mass in the left upper lobe. This mass expanded on deep inspiration and contracted noticeably on the Valsalva maneuver. The effects of respiration and the Valsalva maneuver were recorded by planograms. An electroencephalogram was interpreted as being in keeping with an ordinary epilepsy and was not focal in type. Goldstein has reported a case similar to the first patient. Of the fourteen patients with pulmonary arteriovenous aneurysms reported on, eight seem to have had Osler's disease also, but only one was reported as such. Patients with pulmonary arteriovenous aneurysm reported on have had epilepsy, vertigo, syncope, and attacks of numbness and paresthesias of unexplained variety.

Summary.—Two cases of Osler's disease are presented. Both of the patients had cerebral manifestations and one of them also had a pulmonary arteriovenous aneurysm.

10. FACTORS INFLUENCING PRESSURE IN THE PORTAL VEIN AS MEASURED IN THE INTACT ANIMAL

F. W. HOFFBAUER, M.D., J. L. BOLLMAN, M.D., AND J. H. GRINDLAY, M.D. (BY INVITATION), ROCHESTER, MINN.

The influence of various physiologic factors upon the pressure in the portal vein has been studied in the intact nonanesthetized dog. In the past, most determinations of portal vein pressures have been limited to measurements made in the anesthetized animal during operation. Information as to the effect of exercise, of ingestion of food, and of straining has not been available. A knowl-

edge of such influences may have clinical significance in relation to the problem of portal hypertension. The problems presented by the existence of such collateral venous channels as the gastric and esophageal varicosities in cirrhosis may, in some cases, outweigh the problem presented by the hepatic metabolic alterations.

METHOD

A 24-inch length of flexible polyvinyl chloride plastic tube* of small bore (internal diameter, 2 mm.) is inserted into a branch of the splenic vein at operation. Before the tube is tied in place, it is threaded down the splenic vein until the tip is just within the portal vein. The tube is led to the outside through a stab wound. In order to record changes in intraperitoneal pressure, a thin rubber balloon (capacity, 60 ml.) is placed in the peritoneal cavity. The balloon is filled with water and connected to a similar length of plastic tubing. Pressure in the inferior vena cava has also been determined by a technique similar to that used for the portal vein. A left nephrectomy is performed. A plastic tube is inserted through the renal vein.

The venous cannulae are filled with a citrate solution and the free ends are plugged when readings are not being made. This plastic tubing causes much less reaction in the lumen of the vein than does rubber or glass. Thrombus formation may be delayed by the administration of dicumarol. A free flow of blood from the tube may be obtained for ten to fourteen days, occasionally longer. The time interval is sufficient for complete recovery from the effects of the operation before observations are undertaken. Pressure readings are made in water manometers attached to the side of the dog by means of a light harness. All readings are made with the animal in the standing position.

RESULTS

In normal intact dogs the pressure in the portal vein varies from 4 to 10 cm. of water. The pressure in the inferior vena cava is 0, or occasionally 2 cm. below atmospheric pressure. Exercise produces no significant increase in portal pressure. The straining that accompanies urination, defecation, and vomiting produces sharp increases in the portal pressure. This is associated with increases in intra-abdominal pressure and in inferior vena caval pressure as well.

The ingestion of food produces an increase in portal vein pressure that persists for several hours. Initially this is associated with a rise in intra-abdominal pressure. The latter returns to the original level before the portal vein pressure does. There is a period following the ingestion of food during which the elevation of portal pressure appears to be unopposed by other forces.

Acute liver damage resulting from exposure to carbon tetrachloride vapor does not appear to be associated with a portal hypertension despite the fact that such livers appear congested upon examination. Such animals have shown a normal portal vein pressure response during exercise.

It is hoped that observations by this method in the intact animal may prove useful in studying the relationship of portal vein pressure to development of collateral venous channels and to ascites formation. It should also be possible to evaluate the reduction of portal pressure brought about by surgically created venous shunts in experimental cirrhosis.

*The plastic tubing used in these experiments is manufactured under the trade name "Transflex" and was furnished by the courtesy of Irvington Varnish and Insulator Company, Irvington, N. J.

11. TREATMENT OF ENDOGENOUS HYPOVITAMINEMIA A IN LIVER DISEASE

HANS POPPER, M.D., FREDERICK STEIGMANN, M.D., AND HATTIE A. DYNIEWICZ, PH.C. (BY INVITATION), CHICAGO, ILL.

Previous experiments have shown that the vitamin A metabolism in liver disease is altered in several ways. The release of vitamin A from the liver is disturbed since in acute liver disease, despite considerable hepatic vitamin A stores, the plasma vitamin A drops rapidly. This disturbance in vitamin A release is due to displacement of vitamin A within the liver cells as demonstrated by fluorescent microscopy. Another disturbance is impaired intestinal absorption as indicated by the flat vitamin A tolerance curve after oral administration of vitamin A. The endogenous nature of the hypovitaminemia A in liver disease points, therefore, to the necessity of administering extremely large doses of vitamin A or to correction of the faulty intestinal absorption. Administration of bile acids did not alter the flat tolerance curve.

In more recent experiments the administration of lecithin, wheat germ oil, and a fat-soluble antioxidant did not significantly or regularly raise the tolerance curve in hospital control subjects or in patients with liver disease. The administration of 50 mg. of mixed tocopherols simultaneously or for several days prior to the administration of 75,000 units of vitamin A also did not raise the curve significantly. This was confirmed by experiments on vitamin A-deficient rats in which the hepatic vitamin A concentration after feeding of one dose of vitamin A was not influenced by the simultaneous administration of tocopherol. In contrast, the hepatic vitamin A concentration after administration of repeated doses of vitamin A is far higher in animals receiving supplements of tocopherol. This points to a protecting mechanism of the latter within the liver.

Following recent reports about improved absorption of aqueous vitamin A preparations, the vitamin A tolerance curve was compared after administration of vitamin A in oil and in aqueous solution containing sorbitan monolaurate derivative as dispersing agent and propyl gallate as antioxidant. In twenty hospital control subjects and thirty-six patients with various liver disease (most of them with low plasma vitamin A), the tolerance curve after administration of 30,000, 75,000, or 600,000 units of vitamin A was much higher with the aqueous than with the oily solution, independent of the order of administration. The flat vitamin A tolerance curve in liver disease was thus raised to peaks above those found in hospital control subjects after administration of equal amounts in oil. The administration of aqueous vitamin A preparations therefore appears suitable for compensating the faulty absorption of vitamin A caused by liver damage.

12. STUDIES IN NITROGEN METABOLISM

D. KOZOLL, M.D., AND W. T. MOK, M.D. (BY INVITATION)
CHICAGO, ILL.

Influence of Oral and Intravenous Administration of Amino Acids Upon Nitrogen Balance.—A varied opinion exists as to the relative effectiveness of amino acids given by mouth and by vein, most workers indicating a slight advantage of the latter. Our clinical experience differed, and for that reason a study was carried out in which eight patients were given identical amounts of the same amino acid preparation by vein and by mouth and the nitrogen balance achieved compared. Each patient was kept on a 2,000 calorie diet which

was virtually nitrogen free for the entire period of study. For periods of five days each the patients were given quantities of a lyophilized amino acid preparation* intravenously and then the same quantity was given for another five-day period orally. In three of eight patients, oral administration produced a positive balance at the level of amino acids given, whereas the same quantity intravenously did not. In seven of the eight patients, the nitrogen balance during periods of oral administration was significantly higher than during equivalent periods of intravenous use. In one patient, in whom both routes produced positive balance, that produced by the intravenous route was significantly higher. This evidence indicates the advantages of the enteral route of administration of protein digests wherever possible.

Influence of Caloric Intake Upon Nitrogen Balance.—There has been established a concept that the degree of nitrogen balance will be greatly influenced by the caloric intake. Actually, few studies have investigated this particular point. In five patients a constant quantity of lyophilized amino acid preparation† was administered intravenously daily for five periods; this dose was calculated barely to approach nitrogen equilibrium. During each five-day period, the oral caloric intake, which consisted of a carbohydrate diet, almost free of protein, was varied between 500 and 3,000 calories. It was found necessary to administer a minimum of approximately 1,000 calories per day during each period to produce nitrogen balance. However, in no instance did an increase in the caloric intake beyond 1,000 calories lead to a consistently more positive nitrogen balance. This supports previous observations in which no consistent correlation could be made between the caloric intake of a given patient and the nitrogen balance achieved.

*Provided by the Interchemical Corporation and prepared for intravenous use.
†Interchemical Corporation.

PROGRAM

SCIENTIFIC PROGRAM—OCTOBER 31, 1947

FRIDAY AFTERNOON, 2:00 P.M.

13. PENICILLIN IN THE TREATMENT OF ACTINOMYCOSIS

DONALD R. NICHOLS, M.D., AND WALLACE E. HERRELL, M.D.
ROCHESTER, MINN.

An appraisal of the value of penicillin in the treatment of actinomycosis is reported. The report is based on a review of ninety-eight cases of maxillofacial, pulmonary, abdominal, and pelvic infection. The period of follow-up of these patients varied from one year to five years. Forty-five patients received penicillin; fifty-three patients did not receive it.

Of the group of patients suffering from maxillofacial actinomycosis, twenty-six received penicillin and twenty-five did not. Examination of the results reveals that the number who recovered was approximately equal in both groups (in excess of 90 per cent). However, the period of disability of those patients who received penicillin was markedly shortened. Furthermore, the sinuses healed within a short period after the institution of penicillin therapy.

From the standpoint of comparison between those patients who received penicillin and those who did not, the most striking results occurred in the pulmonary, abdominal, and pelvic infections. Nine patients suffering from pulmonary actinomycosis received penicillin. There were five recoveries and four failures. Of the thirteen patients suffering from pulmonary actinomycosis who were untreated or received treatment other than penicillin, seven died, five still had evidence of disease when last heard from, and only one showed evidence of improvement. Of seven patients with abdominal actinomycosis who were treated with penicillin, five recovered and two did not. Of fourteen patients suffering from abdominal actinomycosis who did not have the benefit of penicillin therapy, nine died, four still had evidence of disease when last heard from, and only one was said to have recovered. There were three patients suffering from actinomycosis involving the pelvic viscera, all of whom recovered after surgical drainage and penicillin therapy. There was one patient with pelvic actinomycosis in whom penicillin was not used. The treatment employed was not effective. Prior to the introduction of penicillin therapy, instances of pelvic actinomycosis in which cures were obtained were, in our experience, rarely if ever encountered.

The diagnosis of actinomycosis in each case was made by means of direct examination or culture of pus or of material obtained at operation. All strains of *Actinomyces bovis* cultured were found to be sensitive to the action of penicillin in vitro.

14. EFFECT OF PULMONARY EDEMA ON SUSCEPTIBILITY OF MICE TO PNEUMOCOCCAL PNEUMONIA

CARL G. HARFORD, M.D., AND MARY N. HARA, A.B. (BY INVITATION)
ST. LOUIS, MO.

Previous experiments have demonstrated that type I pneumococci are eliminated rapidly from the lung of the normal mouse but grow in the lung containing an influenza viral lesion. Histologic examination of the influenza viral lesion in the mouse has shown the presence of intra-alveolar fluid. The object of the present experiments was to determine whether this fluid could serve to explain the observation of growth of pneumococci in the viral lesion.

Advantage was taken of the facts determined by others, that in the normal animal, protein-containing fluids are removed slowly from the alveoli while crystalloids are absorbed within a few minutes. In confirmation of this slow removal, we have demonstrated in tissue sections that normal mouse serum instilled into the alveoli of normal mice will persist there for at least five hours.

Accordingly, normal serum or saline was given by intrabronchial cannula to groups of mice which then were allowed to inhale fine droplets of type I pneumococci and were observed for survival. In another experiment, the pneumococci were suspended in the inoculum of serum or in Locke's solution for control and the number of pneumococci in the left lobe of the lung determined three hours later in poured blood agar plates.

The results of all experiments indicated that the presence of serum in the alveoli rendered the mice more susceptible to fatal pneumococcal infection and interfered with the normal mechanism for elimination of pneumococci.

The manner of action of serum in the alveoli seems likely to be a combination of three factors: (1) interfering with surface phagocytosis, (2) providing a means of spread of pneumococci throughout the lung in the same way as the edema fluid of established pneumococcal pneumonia, and (3) supplying a culture medium for bacterial growth.

The experiments give evidence supporting the theory that the presence of alveolar fluid is an important factor in the mechanism by which the influenza viral lesion lowers resistance of the lung to secondary bacterial infection. The results suggest also that pulmonary edema in man due to any cause may bring about a greater susceptibility to pathogenic bacteria and pneumonia.

15. SURFACE PHAGOCYTOSIS OF TYPE III PNEUMOCOCCUS

W. BARRY WOOD, JR., M.D., AND MARY RUTH SMITH, M.S. (BY INVITATION)
ST. LOUIS, MO.

Surface phagocytosis, a defense reaction of the host unrelated to humoral immunity, has recently been shown to play an important role in the mechanism of recovery in acute bacterial pneumonia. Since type III pneumococcus produces a particularly virulent form of pneumonia, its behavior toward leucocytes has been studied both *in vitro* and in laboratory animals. Although experimental type III pneumonia in rats has been found to resemble type I pneumonia both in regard to pathogenesis and response to chemotherapy, it differs from the latter in that it frequently causes multiple lung abscesses. Also certain strains of type III pneumococcus, when given to mice via the respiratory tract, are less effectively destroyed in the lung and cause a significantly higher fatality rate.

A plausible explanation for the greater virulence of these type III pneumococci is suggested by the fact that they are more resistant to surface phagocytosis than is the type I organism. Evidence has been obtained that the increased resistance to surface phagocytosis is related to the presence of a peculiar outer capsule or "slime layer" possessed only by the highly virulent strains of pneumococcus III. The slime layer stains metachromatically with methylene blue and is present only on the surfaces of young, rapidly multiplying organisms. When the slime layer is lost with age, the bacterial cell loses its resistance to surface phagocytosis.

Of particular interest is the fact that pneumococcus type III loses its slime layer in vivo during chemotherapy. Once the outer layer is lost, the type III organism, like other encapsulated bacteria, is phagocyted by the surface mechanism and is ultimately killed and digested by the phagocytic cells. These findings would appear to explain certain well-established clinical observations concerning type III pneumococcal infections, particularly in regard to virulence and response to chemotherapy.

16. ANTIHYALURONIDASE ACTIVITY IN HUMAN SERUM WHICH INACTIVATES THE HYALURONIDASES OF TYPES I, II, AND VII PNEUMOCOCCI: RISE IN TITER FOLLOWING PNEUMOCOCCUS BACTEREMIA

ROBERT T. THOMPSON, M.D., CINCINNATI, OHIO
(INTRODUCED BY M. A. BLANKENHORN, M.D.)

A preliminary report on this subject was read at these proceedings two years ago. At that time rises in titer of antihyaluronidase activity which antagonized the hyaluronidases of types I, II, and VII pneumococci were reported in serial sera of five patients following pneumococcus bacteremia. These rises in titer occurred eight to twenty days after bacteremia and were not type specific. Further work on this problem, using the mucoprotein clot prevention test (McClean, 1943) as before, is reported here.

Including patients in the previous report, the serial sera of twenty-six patients with primary pneumococcic pneumonia had initial serum taken within three days of admission to the hospital. Results in these patients are reported here to indicate the occurrence of antihyaluronidase activity in the course of pneumococcic pneumonia.

None of seven patients with negative blood culture exhibited more than twofold rise of antihyaluronidase titer. Serial sera of two patients were titrated against the three enzymes, serial sera of four patients were titrated against two enzymes, and serial sera of one patient were titrated against one enzyme. Changes of antihyaluronidase titer found in these fifteen titrations were one-fourth-fold once, one-half-fold four times, no change five times, and twofold five times.

Eight of eleven patients with pneumococcus bacteremia and no purulent complication exhibited rises of antihyaluronidase titer which were fourfold or greater. Serial sera of all these patients were titrated against the three enzymes. Rise of antihyaluronidase titer found in these thirty-three titrations were no change once, twofold seven times, fourfold six times, eightfold eleven times, and sixteenfold eight times.

All of eight patients with pneumococcus bacteremia and purulent complications exhibited rises of antihyaluronidase which were fourfold or greater. Serial sera of all these patients were titrated against the three enzymes. Rises of antihyaluronidase titer found in these twenty-four titrations were fourfold six times, eightfold five times, sixteenfold three times, thirty-twofold three times, and sixty-fourfold or greater seven times.

One patient with mesothelioma of pleura complicated by pneumococcic empyema and another patient with pneumococcic meningitis secondary to otitis media had rises of antihyaluronidase titer.

These rises of antihyaluronidase titer probably signify excessive elaboration of hyaluronidase by the invading pneumococci, since the antigenicity of hyaluronidases has been demonstrated by McClean (1943). Hyaluronidases act as spreading factors (Chain and Duthie, 1939) so that this elaboration of hyaluronidase may explain the excessive morbidity and mortality of bacteremic pneumococcic pneumonia.

17. EXPERIMENTAL AND CLINICAL INVESTIGATIONS ON THE SPECIFIC TREATMENT OF HUMAN BRUCELLOSIS*

WESLEY W. SPINK, M.D., WENDELL H. HALL, M.D. (BY INVITATION), JAMES SHAFFER, M.D.† (BY INVITATION), AND ABRAHAM I. BRAUDIE, M.D. (BY INVITATION), MINNEAPOLIS, MINN.

The fundamental requisite of successful therapy in human brucellosis is that brucella should be eradicated from the tissues. With this principle in view, this report summarizes a series of investigations which have been under way in the laboratories and clinics of the University of Minnesota Hospitals during the past ten years.

An excellent method for rapidly screening potential therapeutic agents has utilized fertilized chicken eggs infected with a virulent culture of *Brucella abortus* via the yolk sac. Uniformly lethal infections were established in seven-day embryos. With the inoculum used, 50 per cent of untreated controls were dead six days after infection, and 100 per cent, nine days after infection. When sulfadiazine was introduced into the sac twenty-four hours after the brucella, 50 per cent survived twelve days or more after infection. When streptomycin was injected, 50 per cent of the embryos survived nine to eleven days. When the embryos were treated with a combination of sulfadiazine and streptomycin, 50 per cent survived nine to eleven days. An important feature of these observations was not only the protection afforded by these agents, but also the results of cultural studies of the tissues. Brucella were recovered from all untreated controls. Sulfadiazine-treated embryos, when sacrificed, revealed brucella present in 90 per cent, while 77 per cent of the streptomycin-treated eggs contained brucella. Embryos receiving sulfadiazine and streptomycin showed brucella present in the tissues in only 13 per cent.

The foregoing data have correlated remarkably well with the results of therapy in patients having active brucellosis. Since an evaluation of treatment in this disease is dependent upon bacteriologically proved cases, studies have been carried out on thirty-five individuals from whose tissues or body fluids

*Aided by a grant from the United States Public Health Service.
†Research Fellow in Chemotherapy under a grant from Commercial Solvents Corporation.

brucella have been isolated. *Br. abortus* was recovered from thirty-three patients and *Brucella melitensis* from two. Sulfonamides, especially sulfadiazine, were used alone in twenty patients, and coincident with treatment complete recovery occurred in only six patients. Seven patients were treated with streptomycin and the results were less satisfactory than those obtained with sulfadiazine. Nine patients have been treated simultaneously with sulfadiazine and streptomycin with quite satisfactory results. One patient with subacute bacterial endocarditis due to *Br. abortus* recovered completely, except for a residual vertigo, and has remained well for four months after treatment. One patient with a severe and disabling spondylitis recovered promptly. The optimum dose of the two agents awaits further studies, but at the present time it is recommended that 0.5 Gm. of streptomycin should be injected intramuscularly every six hours for seven days for a total of 14 grams. Therapy with sulfadiazine is started at the same time with an initial oral dose of 4 Gm. and then 1 Gm. every four hours for two to three weeks. In conclusion, experimental and clinical investigations indicate that the simultaneous use of streptomycin and sulfadiazine will provide a more satisfactory form of specific therapy for brucellosis than has been described to date.

18. SIGNIFICANCE OF COPROANTIBODY EXCRETION DURING ENTERIC INFECTION*

PRESTON E. HARRISON, M.D., PH.D.
HOUSTON, TEXAS

(INTRODUCED BY JAMES A. GREENE, M.D.)

A study was made of fecal antibody excretion from patients with salmonella and shigella infections. Diarrheal and dysenteric stools obtained during the acute infection, recovery, and convalescence were examined for coproantibody simultaneously with culture and serum agglutinin titration. Positive cultures were obtained from 58.6 per cent of the patients and fecal agglutinins were found in 91.3 per cent. Coproantibody for the homologous organism, or for one or more enteric pathogens in those specimens from which a positive culture was not obtained, was demonstrated in high titer on several occasions from each patient. The antibody was present as early as the third day, reached peak titer by the tenth day, rapidly declined as recovery was initiated, and completely disappeared during convalescence. In those cases in which clinical recovery was not obtained, coproantibody persisted throughout the observation period. Circulating antibody did not rise to significant titer until about the time recovery was initiated and then gradually rose to high titer which persisted during the remainder of the observation period. Coproantibody titration provided a measure of efficacy of therapy in that following a good chemotherapeutic response coproantibody titer rapidly declined and disappeared when cultures became negative and after recovery definitely was established. In contrast, coproantibody persisted when clinical recovery was not obtained.

Coproantibody could be demonstrated only during active infection, or clinical activity of disease, and was not present in carriers who showed no evidence of infection. In chronic ulcerative colitis coproantibody for one or more species of shigella, and often for species of salmonella, was present during episodes of exacerbation of symptoms.

*Aided by a grant from the Committee on Scientific Research of the American Medical Association.

19. RESULTS OF VACCINATION AGAINST EPIDEMIC INFLUENZA DURING THE SPRING OF 1947

CLAYTON G. LOOSLI, M.D., PH.D., JAMES A. SCHOENBERGER, M.D. (BY INVITATION), AND G. BARNETT, M.S. (BY INVITATION), CHICAGO, ILL.

In the fall of 1946, a study was organized to test further the prophylactic value of vaccination against epidemic influenza. Two thousand twenty students living in university houses were employed. Seven hundred ninety were vaccinated and 1,230 served as control subjects. Three different commercial influenza vaccines, containing 50 per cent Lee, 25 per cent PR-8, and 25 per cent Weiss strains and prepared by the red cell eluate, calcium phosphate adsorption, and high speed centrifugation methods, respectively, were used. The number of students receiving the three vaccines was approximately the same. Vaccinations, given in the dormitories, were begun November 6 and completed December 6, 1946. The immune response to the three vaccine preparations was approximately the same as determined by the Salk modification of the Hirst chicken red cell inhibition agglutination test.

A sharp increase in the incidence of acute respiratory disease characteristic of mild influenza occurred among the student population during the first week of March, 1947. Antibody determinations for influenza A virus (PR-8 and Weiss strains) showed an increase in titer in convalescent sera from a portion of the unvaccinated patients admitted to the hospital. During the period of increased incidence (March 2 to April 5, 1947), the attack rate was the same (9.5 per cent) in the vaccinated (seventy-five) and unvaccinated (117) groups, with 2.5 per cent (twenty) and 2.36 per cent (twenty-nine), respectively, being admitted to the hospital.

Two strains of influenza virus, found to be closely related, were isolated from throat washings employing the egg technique. The sera of some of the patients admitted to the hospital, both from the vaccinated and unvaccinated groups, showed a marked rise in antibody titer against these strains. Antibody titers for influenza A virus (PR-8) in the acute sera of patients admitted from the vaccinated group were only slightly lower than the postvaccination levels. Sixty pairs of pre- and postvaccination sera were again tested for antibodies against the PR-8, Weiss, and Lee strains of virus as well as the new strains. There was an average of fourfold or greater rise in titer against the former and none against the latter. This shows that there was no close antigenic relationship between the new strains causing the epidemic and those making up the vaccine. This would explain the lack of effect of the vaccine in this outbreak of influenza. The problem of control of epidemic influenza in the light of the foregoing findings is discussed.

20. THE ROLE OF THE ADRENAL GLAND IN IMMUNE MECHANISMS

BENJAMIN C. HOUGHTON, M.D., J. S. THATCHER, M.D., AND CAROLYN HILLES, COLUMBUS, OHIO

(INTRODUCED BY C. A. DOAN, M.D.)

Interest in the relation of the adrenal cortical hormone to the development and release of antibody globulin has been revived in recent years by the work of Dougherty, White, and associates. These authors have reported producing a remarkable augmentation of circulating antibody titers by the administration of

single large doses of adrenal cortical hormone to immunized animals. The anamnestic effect of this hormone exceeded, but was comparable to, the increase in immune titers induced by nonspecific protein and specific antigen administration. Since pituitary adrenotrophic hormone was also effective in this respect, it was suggested that the release of antibodies is a phenomenon mediated by the activity of the adrenal cortex. The concurrent reduction in total lymphatic tissues observed in treated animals led to the conclusion that the dissolution of lymphocytes effected by the concentration of adrenal hormone yields antibody globulin to the component proteins of plasma.

The question advanced by this work relates to the effect of the adrenal cortical function on the (1) formation, (2) release, or (3) distribution of antibody globulin. For the purpose of studying the hormonal effects upon the formation of immune bodies, two groups of cats were employed as experimental animals. One group was given suspended, washed sheep erythrocytes intravenously. The other group was adrenalectomized, maintained on 1 mg. of DOCA daily until adequate equilbrium was established, and then immunized against sheep erythrocytes in an identical manner. Titers of immune body developed in both groups without significant difference. Symptoms of anaphylaxis were exhibited by both groups upon re-exposure to antigen. Sodium ion levels were maintained without change in the adrenalectomized animals throughout the experimental period.

To study the effect of cortical hormone on the release or distribution of antibody globulin, three groups of immunized animals were used. These consisted of normal rabbits, normal cats, and adrenalectomized cats maintained on desoxycorticosterone acetate. Each group was subjected to a large single intravenous injection of whole adrenal cortical extract (Thatcher and Hartman). No augmentation of titers was observed at three, six, nine, or twelve hours, or subsequently. Realizing the possible differences in preparation, refinement, and content of hormone, this experiment was repeated using Wilson's whole cortical extract. Results were identical.

It is apparent, therefore, that the adrenal cortical hormones do not either affect the capacity of the animals to form immune bodies when exposed to antigen nor alter the titers of such immunity when it is established.

Hematologic studies carried out concurrently on normal immunized and adrenalectomized cats revealed wide individual variations in total lymphocyte levels. These levels, high or low, bore no relation to the capacity to form or maintain titers of immunity.

21. INSULIN DEPRESSION AND CARBOHYDRATE EXCITATION OF THE PANCREATIC ISLETS OF LANGERHANS

JEROME W. CONN, M.D., AND STEFAN S. FAJANS, M.D. (BY INVITATION)
ANN ARBOR, MICH.

Patients suffering from periodic spontaneous hypoglycemia produced by secreting islet cell tumors do not all yield the same blood sugar response to a test dose of glucose. About one-half of such patients have responded with a hyperglycemic, plateau (diabetic) type of curve, while the others have shown a normal or subnormal initial elevation of the blood sugar, followed by a rapid fall to hypoglycemic levels. Fasting hypoglycemia has been common to both groups. The discrepancy has aroused abundant speculation.

Our recent studies upon such patients have suggested that: (1) The presence or absence of the diabetic type of curve is dependent upon the functional

state of the islets of the pancreas proper. (2) The functional state of the pancreatic islets is conditioned by the intensity of the hyperinsulinism produced by the abnormal tissue; the more severe the hyperinsulinism, the more likely is pancreatic depression and a diabetic type of curve. (3) The amount of carbohydrate in the antecedent diet exerts an important influence upon the response.

The present experiments were designed to test these tentative conclusions. Dogs on constant diets were chronically hyperinsulinized with large daily doses of protamine zinc insulin for periods of four to eight months. Daily fasting blood sugar was maintained between 20 and 40 mg. per cent. Four-hour glucose tolerance tests were done weekly under a variety of conditions. The same animals were then made diabetic with alloxan, again hyperinsulinized as before, and the same experiments repeated. Differences in results before and after alloxan can be attributed to the presence or absence of the beta cells of the islets of Langerhans.

The results indicate:

1. The secretion of insulin by the pancreas can be greatly inhibited by long-continued administration of exogenous insulin, but sufficient insulin to produce severe, chronic hypoglycemia is required. Under these circumstances the blood sugar response to a test dose of glucose is of the diabetic type, even though there is sufficient steadily absorbed insulin acting during the test to have produced intense and prolonged hypoglycemia when no glucose was given over a similar test interval.

2. A small amount of insulin given intravenously at the height of the diabetic response to glucose produces a precipitous fall of the blood sugar. Thus the ability of the animal to respond normally to a sharp rise of the blood insulin level remains intact.

3. Insulin-inhibition of endogenous insulin production can be overcome in a few days by the administration of large amounts of carbohydrate despite continuation of the inhibiting dose of insulin. The hypoglycemic phase of the tolerance curve is restored.

4. Following alloxan destruction of the beta cells and the reinstitution of hyperinsulinism the blood sugar response to glucose is diabetic in type in the presence of the steady absorption of depot insulin. The precipitous fall of the blood sugar upon intravenous administration of insulin at the height of the blood sugar curve is again obtained. *However*, prolonged administration of carbohydrate in the presence of continued exogenous hyperinsulinism now fails completely to alter the fixed diabetic, plateau response to a test dose of glucose.

5. The former ability of the animal to convert an insulin-induced diabetic response to a normal one under the influence of high carbohydrate feeding is completely lost when the beta cells have been destroyed and are no longer capable of being stimulated to activity by high carbohydrate feeding.

6. The hypoglycemic phase of the glucose tolerance curve is dependent upon an active and rapid release of insulin into the blood stream in response to the physiologic stimulus for such release. This functional capacity of the beta cells can be depressed by exogenous insulin and awakened by high carbohydrate feeding, and the final response (within the limits of these experiments) is dependent upon the relative intensities of the depressing and awakening stimuli acting simultaneously.

22. THE DIFFERENTIATION OF INCIPIENT HYPERTHYROIDISM

ROY E. SWENSON, M.D. (BY INVITATION), AND GEORGE M. CURTIS, M.D.
COLUMBUS, OHIO

The protein-bound blood iodine is of value in recognizing incipient hyperthyroidism associated with endemic goiter. It is a useful diagnostic tool in differentiating other conditions associated with an elevated basal metabolic rate, particularly when goiter is present.

For the region of Central Ohio, the average normal basal metabolic rate is -5 ± 8 per cent, while the average normal protein-bound blood iodine concentration is 0.88 ± 0.2 micrograms per 100 milliliters.

When 178 patients with untreated, uncomplicated nontoxic nodular goiter were grouped according to standard deviations from the average normal basal metabolic rate, it was found that the average protein-bound blood iodine progressively increased with each increment of the basal metabolic rate. A significant number of patients had basal metabolic rates of +3 per cent or above and protein-bound blood iodine concentrations of 1.2 micrograms per cent or above.

Of the uncomplicated cases, 66.8 per cent had symptoms suggestive of hyperthyroidism and of these, 70.6 per cent had elevated protein-bound blood iodine concentrations, while only 47 per cent had elevated basal metabolic rates. The protein-bound blood iodine was a better index of thyroid function than the basal metabolic rate alone. Symptoms listed according to their significant correlation to an elevated protein-bound blood iodine are: a pulse of 90 or more per minute, palpitation, weight loss, weakness, nervousness, and emotional instability. Of those with symptoms, 34.5 per cent had elevated protein-bound blood iodide concentrations associated with basal metabolic rates below +3 per cent. Here the incipient hyperthyroidism was unmasked by determination of the protein-bound blood iodine.

Because of the significantly elevated protein-bound blood iodine and the significant incidence of symptoms suggestive of hyperthyroidism in those patients with basal metabolic rates of +3 per cent or above, it is our conclusion that incipient hyperthyroidism is present in those patients with nodular goiter having (1) Basal metabolic rates of plus 4 per cent or above and protein-bound blood iodine concentrations of 1.2 micrograms per cent or above; (2) symptomatology suggestive of hyperthyroidism and a protein-bound blood iodine concentration of 1.2 micrograms per cent or above.

The basal metabolic rate is frequently elevated in association with certain cardiovascular diseases. When goiter is present, the protein-bound blood iodine is a useful diagnostic aid in evaluating thyroid function.

PROGRAM

SCIENTIFIC PROGRAM—NOVEMBER 1, 1947

SATURDAY MORNING, 9:15 A.M.

23. INHIBITION OF THE PRESSOR RESPONSE TO EPINEPHRINE INJECTION IN COMPLETE ANOXIA

A. Surtshin, M.D. (By Invitation), S. Rodbard, M.D. (By Invitation), and L. N. Katz, M.D., Chicago, Ill.

We have recently observed an unexpected inhibitory effect of complete anoxemia on the pressor response to epinephrine. In the normal dog, an injection of 0.5 mg. of epinephrine intravenously causes a marked rise in arterial pressure which occurs within a few seconds and gradually diminishes during the next few minutes.

In open-chested dogs which are placed on pure nitrogen breathing, the oxygen tension of the blood falls very rapidly from normal to values approaching 0 volumes per cent within ninety seconds. During this period of developing anoxia the blood pressure tends to rise about 40 mm. Hg above the control level. The pressure then begins to fall steadily and this continues until death occurs, unless air breathing is resumed. When air breathing is reinstituted in the control experiments, a sharp postanoxic rise in arterial pressure is observed, this rise being somewhat similar in form to that seen after epinephrine injection in control animals.

The injection of 0.5 mg. epinephrine intravenously during the period of rising blood pressure in developing anoxia results in only a markedly diminished and very transitory rise in arterial pressure. If the anoxia is continued, the pressure falls as in the control anoxia experiments. If the injection is given after the pressure has started to fall in the advanced anoxic stage, epinephrine has no pressor effect and the late anoxic pressure decline continues. Reinstitution of air breathing now results after a brief delay in a typical epinephrine effect with the pressure rising to higher levels than those seen in the control post-anoxia rise.

These results suggest that in conditions of extreme anoxia such as might be seen in drowning or other forms of extreme asphyxia, the injection of epinephrine may lead to no change in arterial pressure inasmuch as the cardiovascular system seems to be incapable of responding to this pressor agent. As a matter of fact, our results show that under these conditions of extreme anoxia, endogenous pressor states exist which, upon the administration of oxygen in adequate amounts, would in themselves cause a marked rise in arterial pressure.

24. THE ROLE OF ARTERIAL PRESSURE CHANGES IN THE INDUCTION OF EPINEPHRINE-CYCLOPROPANE IDIOVENTRICULAR RHYTHMS

G. K. Moe, M.D., S. D. Malton, M.D. (By Invitation), Walter Freyburger, B.S. (By Invitation), and Barbara Rennick, M.S. (By Invitation)
Ann Arbor, Mich.

Ventricular extrasystoles, ventricular tachycardia, or ventricular fibrillation can be regularly produced in dogs under cyclopropane anesthesia by doses of epinephrine ranging from 1.0 to 10 micrograms per kilogram. Although the exact mechanism is unknown, it has been observed that dibenzyl-B-chlorethylamine (Dibenamine), which "reverses" the pressor action of epinephrine but does not abolish its chronotropic and inotropic actions, protects the heart against epinephrine-induced idioventricular rhythms. We have been able to demonstrate that Dibenamine prevents ventricular ectopic rhythms *because* it prevents the pressor response to epinephrine.

In dogs, doses of epinephrine which regularly produced ventricular automatism in the control periods failed to do so after the intravenous injection of 15 to 20 mg. per kilogram of Dibenamine. Clamping of the thoracic aorta, which raised arterial pressure by 50 to 100 mm. Hg, also failed to provoke idioventricular discharges in the Dibenaminized animal, but when small doses of epinephrine were followed within ten seconds by aortic occlusion, ventricular tachycardia resulted. After larger doses of epinephrine, which alone failed to arouse ectopic foci, ventricular tachycardia could be induced repeatedly by occlusion, and terminated by release, of the aorta.

In other experiments without Dibenamine, arterial pressure was controlled by means of a pressure stabilizer attached to the abdominal aorta. Doses of epinephrine which regularly established ventricular foci when the stabilizer was clamped off failed to do so when the pressor response was prevented by opening the stabilizer. For example, in one experiment 2 micrograms per kilogram caused a rise of pressure and ventricular tachycardia in control trials with stabilizer closed, but 16 micrograms per kilogram failed to disturb the normal sinus rhythm with the stabilizer open.

Although a rise of arterial pressure greatly facilitates the induction of idioventricular activity by epinephrine, the direct accelerator action of the drug is also important, for elevation of pressure alone will not usually produce more than occasional ventricular extrasystoles associated with reflex vagal slowing of the sinus node.

25. AXIS DEVIATION IN HUMAN BUNDLE BRANCH BLOCK

A. Morgan Jones, M.R.C.P.* (By Invitation), Manchester, Eng., and Harold Feil, M.D., Cleveland, Ohio

We have noticed that, in a few cases in which bundle branch block appeared while the patient was under observation, there was little change either in the axis deviation or in the general pattern of the electrocardiogram. This study is an attempt to ascertain how far the axis deviation associated with bundle branch block is determined by the pre-existing electrical axis with normal conduction. From fifty-four patients, in whom both normal conduction and bundle branch block had been recorded, we were able to collect 192 records, ninety showing only bundle branch block, ninety-two showing only normal con-

*Rockefeller Travelling Fellow.

duction, and ten showing both bundle branch block and normal conduction complexes in all limb leads. In thirty-seven patients adequate chest leads indicated the side of the block, but in the remaining seventeen the side of the block was judged by the limb leads; there were thirty-three patients with left bundle branch block and twenty-one with right. The average position of the electrical axis with normal conduction and with bundle branch block was determined in each case by averaging the axis position in all available records with one type of intraventricular conduction.

In thirty-three patients who developed left bundle branch block, the average shift of the electrical axis associated with the block was 12 degrees to the left; in twenty-one patients who developed right bundle branch block the average shift of axis was 12 degrees to the right. In only twelve patients developing left and ten developing right bundle branch block was the axis shift 15 degrees or more. In the majority of instances the general patterns of the electrocardiogram was closely similar before and after the development of the block. Although there may have been changes in the electrical axis (due to alteration in the position of the heart, ventricular hypertrophy, cardiac infarction, or pulmonary embolism) between the normal conduction and bundle branch block records, these factors could have affected the apparent change of axis in either direction; there is, therefore, no reason to assume that they materially affected the average results, although they must be taken into consideration in individual cases. These extraneous factors can be excluded in the eight cases with normal conduction and bundle branch block complexes in the same record; the results in these cases were similar to those in the whole series.

It is concluded that the axis deviation and the general pattern of the electrocardiogram are not greatly modified by the appearance of bundle branch block and that the axis deviation associated with right and left bundle branch block is due principally to the position of the mean electrical axis of QRS before the bundle branch block appeared.

26. REGIONAL PRODUCTION OF MYOCARDIAL NECROSIS WITH ELECTROCARDIOGRAPHIC ANALYSIS

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Chicago, Ill.

(Introduced by G. M. Hass, M.D.)

A new method has been used in producing myocardial lesions, resembling infarcts, in the heart of dogs. The heart is exposed surgically and a hypothermal instrument is applied to a selected area of the epicardium. Lesions, cylindrical in shape, and of controlled dimensions, are then produced by cooling the adjacent myocardium to a low temperature. Lesions of selected dimensions are reproducible in successive animals. Variations encountered in attempts by others to produce lesions by arterial ligation are never observed. Lesions have been repeatedly reproduced in several desired locations in the walls of the auricles and ventricles and in the interventricular septum.

Serial electrocardiographic tracings, using standard limb leads and varied precordial leads, have been obtained at brief intervals. Animals have been sacrificed at various stages in the healing of the lesions up to six weeks, beyond which time all electrocardiographic evidence of cardiac damage has disappeared.

Electrocardiographic analysis indicates that localization of lesions is often inaccurate when standard limb leads and precordial Lead IV are used. These leads are only capable of distinguishing between anterior or apical ventricular lesions and posterior ventricular lesions. Lesions of the right ventricle cannot

be distinguished from those of the left ventricle. The use of standard limb leads and a series of leads from the right and left of the midsternal line has resulted in accurate distinction between lesions of the right and left ventricles, either anteriorly or posteriorly. Evidence of myocardial damage is most pronounced when precordial leads are closest to the lesion. The more distant the leads, the less obvious the changes become. This rule holds only for the first three days. Thereafter, it becomes impossible, even by use of multiple parasternal precordial leads, to distinguish between lesions of the right and left ventricle.

It has not yet been possible to localize large lesions of the auricles or interventricular septum, nor have persistent disturbances in conduction been produced.

27. DICUMAROL IN EXPERIMENTAL MYOCARDIAL INFARCTION

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In a discussion of possible unfavorable side-effects of dicumarol in the treatment of myocardial infarction, the question was raised whether the hemorrhagic stage of the infarct might be more extensive as a result of the altered clotted activity of the blood. This study was undertaken to investigate the influence of dicumarol on the healing of experimental myocardial infarcts. The anterior descending branch of the left coronary artery was ligated in thirty-two dogs. Seven of these died within twenty-four hours. Dicumarol was administered to sixteen in doses sufficient to maintain the clotting activity of oxalated plasma between 40 and 25 per cent of normal. The remaining nine dogs were controls. The animals in each group were sacrificed from five to twenty-one days after the occlusion of the coronary artery. On gross examination there was no significant difference in the appearance of the infarcts at any stage in the two groups. Mural thrombosis was not found in any heart. Microscopic examination of representative sections did not disclose any obvious difference between the healing infarct in the treated dogs and the controls. Serial electrocardiograms showed no variation in the rate or the character of the evolution of changes associated with anterior infarction in dogs. The sedimentation rate of dogs' blood after coronary occlusion does not follow any consistent pattern, and no influence of dicumarol could be shown by it. Pleural and pericardial adhesions were present to about the same extent in each group of animals.

It is concluded that dicumarol therapy does not have any demonstrable deleterious influence on the healing of experimental myocardial infarction in dogs.

28. EXPERIMENTAL PULMONARY INFARCTION: THE ROLE PLAYED BY ABNORMAL PULMONARY CIRCULATION IN THE PRODUCTION OF INFARCTION BY EMBOLUS

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Houston, Texas

(Introduced by James A. Greene, M.D.)

Pulmonary infarction has never been produced consistently in experimental animals. Numerous investigators have found that obstruction of branches of the pulmonary artery is not in itself sufficient to result in infarction, if in-

farction is defined to include necrosis of lung tissue. We have produced pulmonary embolus in twelve normal dogs by the release of venous thrombi and have produced pulmonary congestion in four dogs by the employment of alpha-naphtha-thiourea; pulmonary emboli and congestion were produced by combining these methods in eight dogs.

No infarction resulted in the twelve normal dogs in which intravascular clots were released. Pulmonary congestion was produced in the four dogs receiving alpha-naphtha-thiourea alone, but no gross or microscopic evidence of pulmonary infarction was demonstrable. Of the eight dogs which received alpha-naphtha-thiourea and emboli simultaneously, three developed pulmonary infarction distal to the emboli, and four showed, in addition, hemorrhage distal to the emboli. Apparently, obstruction of a pulmonary artery or one of its branches by an embolus does not cause infarction of the lungs distal to the obstruction because the blood supplied by the bronchial arteries is adequate to maintain nutrition. It appears, therefore, that if the bronchial circulation is interfered with by pulmonary edema or congestion, alveolar necrosis (infarction) results.

29. STUDIES ON VASOMOTOR TONE IN THE HYPERTENSIVE STATE*

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Since in arterial hypertension the cardiac output is not usually decreased, it follows that there is an increase in total peripheral resistance. The locus of this increased resistance and its "neurogenic" or "humoral" nature are central problems in a study of the pathogenesis of this disease. The circulatory response to the autonomic blocking agent, tetraethylammonium, can be used in an attempt to quantitate the degree to which vascular resistance is dependent on vasomotor tone in the human subject since we have shown by plethysmographic studies that (1) the drug reduces resistance to blood flow in the limbs to the greatest extent in those areas which can be shown by paravertebral sympathetic block to be under greateset vasomotor tone, (2) reduction of vasomotor tone to a limb by body heating reduces the response to the drug, and (3) after sympathetic denervation of an extremity, peripheral vascular resistance is no longer altered by the drug.

Assuming, therefore, that decreases in vascular resistance after tetraethylammonium are in general proportional to the initial level of vasomotor tone, a comparative study was made of the effects of comparable doses of the drug on the resistance to blood flow in the feet and forearms of twelve hypertensive and twelve normotensive subjects. Resistance was calculated from the formula R = mean blood pressure (mm. Hg)/blood flow (ml./100 ml. limb/minute). Reductions in resistance were equal in the two groups, thus suggesting that there was no increase in vasomotor tone in the extremities of hypertensive subjects.

The effects of the drug on the renal circulation were also investigated. Here it could be shown that in certain hypertensive subjects renal blood flow

*Aided by grants from Parke, Davis & Company, The United States Public Health Service, and the Life Insurance Medical Research Fund.

was maintained despite a fall in blood pressure. It would thus appear that increased vasomotor tone may play a significant role in the increased renal resistance of certain hypertensive subjects.

Since blood flow to other vascular areas is less susceptible to accurate measurement, conclusions regarding vasomotor tone from blood pressure observations alone must be accepted with some reservations since not only changes in peripheral resistance but also changes in pulse rate, arterial elasticity, and cardiac output may play a role in the depressor response to the drug. Two lines of evidence would indicate, however, that in other vascular areas than the extremities increased vasoconstrictor tone may be present in the hypertensive subject: (1) After exclusion of both lower legs and one upper extremity from the circulation by arterial tourniquets, the injection of tetraethylammonium still produces a blood presssure fall in hypertensive subjects but rarely in normotensive subjects; (2) after supradiaphragmatic splanchnicectomy the average response of the blood pressure to an injection of the drug is materially decreased.

These studies would seem to provide evidence that in some hypertensive subjects there is an increase in neurogenic vasomotor tone to the splanchnic or renal vascular beds but not to the blood vessels of the extremities.

30. THE EFFECT OF TETRAETHYLAMMONIUM CHLORIDE ON THE BLOOD FLOW IN THE EXTREMITIES OF NORMAL INDIVIDUALS

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The effect of tetraethylammonium chloride on the blood flow in the extremities of seven normal individuals (four male and three female) was studied by the use of an air displacement plethysmograph with a compensating spirometer recorder. Tetraethylammonium chloride was administered to each individual intravenously in amounts of 300 to 400 mg. at the rate of 100 mg. per minute. All experiments were conducted in room temperatures which ranged from 80 to 85° F. (26.6 to 29.4° C.), but no single experiment was done in which there was a variation of more than 1° F. (1.8° C.) in room temperature.

With the subjects lying on the test bed, the plethysmographs were applied to the forearms and legs. The arm plethysmograph included the hand and forearm up to one inch above the olecranon process, and the leg plethysmograph included the foot and leg up to one inch below the tibial tuberosity. After the subject was well adjusted to the surroundings, determinations of blood flow were made for a period of about thirty minutes in order to establish a control level. The drug was then injected intravenously and records of the blood flow were taken for thirty to forty-five minutes thereafter.

The subjective symptoms, after administration of the drug, were a metallic taste, numbness and tingling, tachycardia, dryness of the mouth, and variable disturbances of vision with impairment of accommodation.

The average blood flow in the forearms of all seven subjects before the administration of tetraethylammonium chloride was 4.3 ml. per 100 ml. of limb volume per minute (range, from 1.7 to 7.3 ml.). After the injection of tetraethylammonium chloride the blood flow increased in every case, with a maximum increase occurring between five and fifteen minutes after injection (average about nine minutes). In all seven cases the average maximal blood flow in the forearms, after the injection of tetraethylammonium chloride, was 10.1 ml. per 100 ml. of forearm volume per minute (range, from 3.4 to 14.7 ml.). This rep-

resents an increase of 87 per cent in the average over the control flow. The average control blood flow in the legs of all seven subjects before administration of tetraethylammonium chloride was 3.2 ml. per 100 ml. of limb volume per minute (range, from 1.7 to 4.5 ml.). After injection of the drug the blood flow in the legs also increased in every case and reached a maximum at the same time as in the arms. The average maximal blood flow in the legs, after injection of tetraethylammonium chloride, was 6.9 ml. per 100 ml. of leg volume per minute (range, from 4.0 to 9.5 ml.). This represents an increase of 116 per cent over the control value (range, from 60 to 200 per cent). The increased blood flow in both upper and lower extremities gradually regressed; nevertheless, at the termination of the experiment (thirty to forty-five minutes after the administration of tetraethylammonium chloride), the flow still was higher than the control value. Accompanying the increase in blood flow there was a rise in cutaneous temperature.

31. THE NATURE OF THE COLD PRESSOR TEST AND ITS SIGNIFICANCE IN RELATION TO NEUROGENIC AND HUMORAL MECHANISMS IN HYPERTENSION

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Previous evidence suggests that both neurogenic (autonomic) and humoral factors are concerned in the pathogenesis of increased arteriolar tone in clinical hypertension, but the exact nature of such mechanisms and their relative importance and interrelationships are not well understood. The present study is concerned with the effector mechanism of the cold pressor response in hypertension, utilizing varying degrees of sympathetic block induced by spinal anesthesia and tetraethylammonium chloride. The results clarify the effector mechanism of the cold pressor test and indicate that its utilization along with other procedures may be helpful in evaluating the relative importance of neurogenic and humoral components in patients with this disease.

In each of twenty hypertensive patients the cold pressor response was elicited in the usual manner and then repeated after the administration of tetraethylammonium chloride. One additional patient was tested in a similar manner four months after partial sympathectomy. In eight cases the response was again tested after high spinal anesthesia and in five of these repeated as the anesthesia receded to lower levels. In five instances the effect of tetraethylammonium chloride, was again tested following the performance of the test under spinal anesthesia.

Two phases of the blood pressure response to cold were noted: (1) the response during the minute of exposure to cold (cold minute), the classical cold pressor response, and (2) delayed pressor responses after the hand had been removed from cold water. In all cases tetraethylammonium chloride abolished the cold minute response, demonstrating that the effector phase of this reflex is neurogenic in character and indicating that the cold pressor response may be useful in evaluating procedures designed to eliminate autonomic tone in hypertension. Release of varying portions of the arteriolar bed from active vasomotor control (by spinal anesthesia) produced dampening of the response which tended to vary directly with the extent of denervation, but the smallest portion of the arteriolar bed which, under active vasomotor control, was capable of exciting clinically significant cold pressor reactivity varied from patient to patient.

In one case, who showed marked anxiety and resentment in response to the cold pressor test, there occurred following the cold minute exposure (during tetraethylammonium chloride action) a very sharp transient rise in pressure. Similar, but less marked, delayed transient elevations were seen in eleven other patients. Since such a pressor response occurs at a time when autonomic impulses are blocked at the ganglia, it must be the result of a humoral agent (the action of such agents not being eliminated by tetraethylammonium chloride).

Six (30 per cent) of the twenty patients failed to obtain a depressor effect from the administration of tetraethylammonium chloride, yet the cold pressor response was eliminated in each, suggesting that circulation of a humoral pressor agent, rather than failure of the drug to produce satisfactory autonomic blockade, was responsible for the absence of depressor response in these patients.

The data suggest that both humoral and neurogenic (autonomic) mechanisms may interact as clinically important factors in hypertension.

32. CIRCULATORY RESPONSES TO SPINAL AND CAUDAL ANESTHESIA IN HYPERTENSION: RELATION TO THE EFFECT OF SYMPATHECTOMY*

II. Effect of Renal Function

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In 1944 we found that the renal effects of high spinal anesthesia in patients with essential hypertension were variable. We suggested that the type of response of the renal vasculature might mirror the nature of the hypertensive process. Renal vasodilatation might then be taken as evidence of pre-existing increased vasomotor tone and failure of such vasodilatation as evidence of humoral vasoconstriction or of vascular rigidity due to renal arteriosclerosis. It was intimated that those patients who had predominance of vasomotor influence might receive more benefit from sympathectomy than would others.

The present report includes observations on the renal functional effects of high spinal anesthesia administered to seventeen patients with essential hypertension and of caudal anesthesia given to eighteen patients. Fourteen of the latter group had lumbodorsal sympathectomy.

The effects of spinal and caudal anesthesia upon renal hemodynamics were similar. Reduction of the average arterial pressure of hypertensive patients by 20 mm. Hg, to levels ranging from 85 to 125 mm. Hg, usually causes renal vasodilation, resulting in increased renal blood flow and a slight decrease in glomerular filtration rate.

Qualitatively the renal vascular response of normotensive and hypertensive partients to these procedures is identical. Quantitatively, they may differ slightly in that in hypertensive patients a fraction of afferent arteriolar resistance is not removed by anesthesia.

The renal vasodilator response to anesthesia in hypertension indicates that a large proportion of increased renal vascular resistance in this disease is dependent on nervous influences which are affected by anesthesia extending to about D 5, or alternatively that denervation by anesthesia has sensitized the vessels to vasodilator influences.

The major hemodynamic change induced by spinal or caudal anesthesia is seen to be due to decreased peripheral resistance.

*From the Research Division of the Cleveland Clinic Foundation.

Exceptionally, a few hypertensive patients show deficient or slowed renal vasodilator responses to spinal or caudal anesthesia. Two such patients failed to benefit from lumbodorsal sympathetic ganglionectomy, but operation was equally ineffective in patients whose responses were vasodilator. The renal vascular response to anesthesia is not a positive guide in the selection of patients, although the absence of vasodilation during anesthesia may contraindicate operation.

33. RELIEF OF CAUSALGIA BY USE OF TETRAETHYLAMMONIUM CHLORIDE

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Tetraethylammonium chloride was used for sympathetic blockade to relieve pain in seventeen patients with causalgia. The patients were selected on the basis of complaint of pain without consideration of trophic or vasomotor disturbances. In twelve patients pain was severe enough to prevent proper physiotherapy. Tetraethylammonium chloride was used primarily to obtain symptomatic relief.

Five patients with Sudeck's primary bone atrophy were given this drug in doses of 200 to 500 milligrams. This was sufficient to maintain comfort and to permit physiotherapy. Relief from pain appeared promptly and lasted several hours to forty-eight hours, even though complete sympathetic blockade was not produced. Follow-up clinical and radiologic studies showed improvement in the bone atrophy.

Two patients who were bedridden because of acute osteoporosis of the spine received relief from tetraethylammonium chloride which was comparable to that afforded by the use of narcotics. One of these patients was given 500 mg. to 2.0 Gm. in divided doses daily. She became ambulatory immediately with the additional aid of an orthopedic corset.

Three patients with bilateral thrombophlebitis whose walking was limited by cramps in the legs received extensive therapy with this drug. The relief from pain was not striking, although large doses were beneficial for short periods. The therapy did seem to help somewhat in restoring these patients to full activity.

Tetraethylammonium chloride was given to five patients with causalgia in the presence of known orthopedic disorders. These patients were selected because their pain seemed unrelated and disproportionate to the structural lesion. The bizarre pain was relieved, but the legitimate pain persisted. In two of these patients, swelling of the involved extremities decreased after tetraethylammonium chloride was given.

One patient with multiple pelvic fractures with bilateral pain in the legs had prompt relief of pain lasting about six hours after 225 mg. of the drug. This remission of pain permitted her to move the legs for the first time in nine weeks. Narcotics were required much less frequently thereafter.

A young woman with advanced scleroderma who had pain on motion of the fingers was able to use her hands with much less discomfort after moderate doses of the drug, but there was no significant change in the underlying disorder.

Tetraethylammonium chloride is of definite value in relief of causalgia. The relief of pain may be striking even though the sympathetic blockade is not complete enough to produce increased warmth and dryness of the extremities. The relief is usually sufficient to permit appropriate physiotherapy.

34. THE EFFECT OF DITHIOPROPANOL (BAL) ON HUMAN LEAD INTOXICATION

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Evidence has been accumulating in support of the hypothesis that certain heavy metals are toxic to biologic systems because of their reversible combination with the sulfhydryl groups of the protein fraction of cellular enzymes. There is some evidence that lead may be included in the group of metals that behave in this manner. Barron and Kalnitsky have shown that dithiopropanol (BAL) is one sulfhydryl-containing compound which is capable of reversing such an enzymatic inactivation due to lead. On the other hand, Braun, Lusky, and Calvery felt that lead and dithiopropanol exerted additive toxic effects in lead poisoning as induced experimentally in rabbits.

We have studied thirty men with acute episodes of lead poisoning, ten of whom were treated with dithiopropanol to the limit of tolerance. The drug has no dramatic immediate effect on the symptoms of lead poisoning. As a group the treated men had no different clinical course than the group of untreated men, except for the characteristic local and general effects of dithiopropanol and the occasional production, after prolonged therapy, of generalized muscular aching.

In contrast to the absence of clinical effect, the drug had unprecedented metabolic effects. Characteristically, the cellular lead concentration dropped sharply within a few minutes after the administration of BAL, continued to fall for an hour or longer, and returned to the previous level within twenty-four hours. The urinary lead concentration increased from several to fiftyfold within an hour or so. On the other hand, the lead concentration in the blood plasma of all the men was of a uniform order of magnitude, whether or not they had had abnormal lead exposure, clinical lead poisoning, or dithiopropanol therapy. The treatment did not change the fecal lead concentration or output.

While the changes in the lead concentration in the erythrocyte and urine were obtained again and again following repetitive administration of the agent, they were transient, and not enough lead was removed by the therapy to increase materially the total amount of lead eliminated by the subjects during the period of intermittent therapy or in the weeks that followed.

In summary we have observed that dithiopropanol is of no value in the treatment of acute poisoning in adults due to inorganic lead. We have observed also that the drug, in contrast to the lack of clinical effect on the symptoms of poisoning, causes a transient decrease in the erythrocyte lead concentration and a temporary increase in the urine lead concentration to a degree we have not seen with other types of therapy.

From these two observations we infer that the symptoms and signs of lead poisoning and the storage and elimination of lead may be due to mechanisms that are not intimately related. Our clinical experience in the lead trades supports this inference. The observed effects on lead metabolism are consistent with the hypothesis that lead combines with an erythrocyte component and that this combination can be reversed in vivo by dithiopropanol therapy, but the failure of symptomatic response to therapy does not give support to the theory that this particular enzyme system is involved in the mechanism of lead poisoning.

TO BE READ BY TITLE

35. CONGENITAL FAMILIAL HYPOPROTHROMBINEMIAS

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The experimental evidence of the writer that prothrombin is a complex composed of several components suggests the possibility that more than one type of hypoprothrombinemia may occur clinically. This was borne out by studies of two families, in each of which several members have a hypoprothrombinemia. In the first family the mother, one son, and one daughter have a prothrombin time of 15½ to 16 seconds. On mixing their plasmas with an equal volume of normal plasma, a mixture is obtained which has a prothrombin time of 13½ seconds, the expected calculated value based on the writer's prothrombin curve. This type of hypoprothrombinemia is similar or identical with that produced by dicumarol poisoning. In this type of hypoprothrombinemia, it is component B which is reduced.

In the second family, two brothers are affected. Each has a prothrombin time of 19 to 20 seconds. Both have a bleeding tendency. When their plasmas are mixed with an equal volume of normal plasma, a normal prothrombin time (12 seconds) characteristically results. This same behavior has been noted with plasma from patients with avitaminosis K. When plasma from a member of family I is mixed with an equal volume of plasma from a member of family II, a prothrombin time of 13½ seconds results, thus showing that the second plasma has the same corrective capacity on the first as normal plasma. In other words, the first plasma contains an excess of the factor lacking in the second plasma (component A), whereas the second plasma contains as much component B as normal plasma.

The prolonged prothrombin time resulting when oxalated plasma is stored is due to the disappearance of a principle that is neither component A nor B. It is not adsorbed by $Al(OH)_3$, $Ca_3(PO_4)_2$ and similar agents. This substance has been tentatively named the labile factor. Its lack in plasma may apparently cause a hemorrhagic diathesis as the recent report of Owren suggests.

The prothrombin time serves as a reliable guide of hemostatic efficiency even though it is dependent upon three factors: components A and B and the labile principle. A hemorrhagic tendency appears when the prothrombin time is 19 seconds or longer.

36. THE BLOOD COAGULATION-RETRACTION TIME AND ITS RELATION TO THROMBOSIS*

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Clot retraction is dependent upon thrombocytes, surface forces, erythrocyte mass, and qualitative and quantitative variations in fibrin. The same factors are also important elements in the development of the thrombotic tendency in individuals.

*Supported by a Grant-in-aid from the United States Public Health Service.

A method which measures the combined coagulation and clot retraction time of capillary blood was used in the study of 100 normal individuals, 100 postoperative patients, and 300 hospitalized medical patients. The coagulation-retraction time is determined by suspending a 20 c.mm. drop of capillary blood in castor oil. The drop is observed at five-minute intervals for the development of a bud of serum which projects into the oil from the retracting clot. The normal coagulation-retraction time is longer than 20 minutes, the average for 100 individuals being 33.1 minutes.

Patients in the postoperative period have shortened coagulation-retraction times. Approximately one-third of these patients had coagulation-retraction times less than 20 minutes during most of the postoperative period. Of the 300 general medical patients, 41 per cent had coagulation-retraction times less than 20 minutes and 11 per cent less than 15 minutes. One patient in this group developed pulmonary embolism when her coagulation-retraction time was 11 minutes (the shortest time observed in over 3,000 determinations).

The coagulation-retraction test is well adapted to the control of heparin therapy and can be used instead of the ordinary coagulation time determinations. The test is not reliable in following dicumarol therapy because its correlation with prothrombin time is not accurate. It is suggested that patients whose coagulation-retraction time is consistently less than 20 minutes be given prophylactic anticoagulant therapy.

37. THE EFFECT OF FOLIC ACID ON RADIATION INDUCED ANEMIA AND LEUCOPENIA

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Penetrating radiations originating external to the body and certain radioactive elements deposited within the body produce anemia, leucopenia, and other effects on the blood and blood-forming tissues when given in adequate doses. It is of interest that certain species develop a striking increase in mean corpuscular volume concomitant with the anemia after exposure to these physical agents. Experiments have been conducted on the human, rabbit, and rat in which attempts were made to modify these hematologic effects of radiations by the enteral and parenteral administration of folic acid. The penetrating radiation used in these studies was confined to x-ray (200 kv.) and was administered to the whole body in single (rabbits) or divided doses (human beings). The radioactive elements used were P^{32} (human beings) and Sr^{89} (rats) given intravenously in the former and intraperitoneally in the latter subjects. In certain of these experiments the folic acid was given before the radiation or radioelement was administered and continued. In others the folic acid was begun approximately at the point of maximum anemia or leucopenia or both and continued.

The anemias and leucopenias induced by these radiations were not prevented nor was recovery hastened by the administration of folic acid in adequate doses. The macrocytic anemia produced in rats by radiostrontium and in human beings by total body x-radiation or radiophosphorus was likewise unaffected.

38. COMBINED SYSTEM DISEASE AND HEMATOLOGIC RELAPSE OCCURRING IN PERSONS WITH PERNICIOUS ANEMIA TREATED WITH SYNTHETIC FOLIC ACID (PTEROYL GLUTAMIC ACID) FOR A PERIOD OF TWO YEARS

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The observations presented have evolved from an extension and elaboration of studies undertaken in November, 1945, and reported at this meeting one year ago. Elucidation of the following questions has been sought: (1) Will synthetic folic acid given orally or parenterally control the anemia and glossitis of pernicious anemia and sprue for long periods of time as successfully as liver extract? (2) Will synthetic folic acid therapy prevent the development of the neurologic manifestations of pernicious anemia or control them after they have appeared?

Twenty-eight persons with pernicious anemia, the majority of whom had been controlled for two to seventeen years by injections of liver extract, were treated with folic acid orally for six months to two years. Folic acid treatment of twenty-four of these was begun on or about November, 1945; the remaining four have been treated six to eighteen months. Oral doses ranged from 30 mg. three times a week to 50 mg. daily.

Nine patients with pernicious anemia, six of whom were in relapse and three of whom previously had been maintained on parenteral liver extract, were treated with folic acid parenterally for six to twelve months. Doses ranged from 30 mg. daily to 30 mg. weekly. Three persons with sprue and one with macrocytic anemia secondary to ileosigmoidostomy were observed for nineteen to twenty-five months while receiving varying doses of folic acid orally. Complete physical and neurological examinations, erythrocyte counts, hemoglobin levels, hematocrit determination, and reticulocyte counts were performed at the beginning of the study and were repeated as often as the condition of the patient demanded.

So far, combined system disease has occurred in eight of the thirty-seven persons with pernicious anemia. During the first year four of the patients given folic acid orally developed posterolateral sclerosis after five to eight months of therapy. One of these showed mental deterioration. In the second year three patients on oral and one on parenteral therapy developed signs of posterolateral sclerosis, two with distinct mental changes, within four to eighteen months of the beginning of treatment. Satisfactory remissions followed the administration of liver extract intramuscularly.

Hematologic values have neither reached nor been maintained at optimum levels in the nine persons treated parenterally and one-third of the persons receiving folic acid orally developed moderate degrees of anemia. Gastric carcinoma and unexplained rectal bleeding accounted for the anemia in two of these. One patient had a striking hematologic and neurologic relapse after eighteen months of oral thearpy (30 mg. three times a week). After intramuscular folic acid therapy for two weeks (30 mg. three times a week) the relapse continued unchecked. Erythrocytes had fallen to 1.51 millions and hemoglobin to 7.2 grams. Urine assays indicated that 50 to 80 per cent of the folic acid administered was excreted in the urine within twenty-four hours. The patient was given 12 Gm. of 5-methyl uracil daily for ten days. Reticulocytes rose to 10 per cent on the fourth day of this treatment and there was a

subsequent rise in red cells and hemoglobin to 2.45 millions and 9.5 grams. After discontinuance of 5-methyl uracil, complete hematologic and partial neurologic remission was achieved by parenteral liver therapy. Distinct glossitis occurred in five of the patients with pernicious anemia during folic acid therapy and waxed and waned without relation to the administration of the drug. The persons with sprue and secondary macrocytic anemia were maintained in better clinical and hematologic states than had resulted from liver extract therapy.

In conclusion, the second year of this study demonstrates that folic acid will not maintain hematologic remissions in all patients with pernicious anemia nor will it prevent the appearance of glossitis. In addition it confirms the previous observations that posterolateral column disease and mental deterioration may develop in persons with pernicious anemia even though their hematologic state is well controlled by folic acid.

39. TREATMENT OF PERNICIOUS ANEMIA WITH PTEROYL GLUTAMIC ACID (FOLIC ACID)

STEVEN O. SCHWARTZ, M.D., AND BERTHE E. ARMSTRONG, M.D., (BY INVITATION), CHICAGO, ILL.

The paper is a résumé of experiences with folic acid in the treatment of pernicious anemia. The studies were begun in January, 1946, and over 100 patients have been maintained for varying periods of time on 5 mg. doses (daily). The discussion will center on such patients as have been either taken off the drug or maintained for twelve months or longer. In this category are sixty-nine patients. Of these, thirty-eight were men and thirty-one were women. The ages varied from 29 to 80 years. Six were placed on folic acid therapy immediately after an "assay period," while sixty-three were changed from routine maintenance liver therapy. Fifty-two are still on folic acid (as of September, 1947), four have disappeared from observation, and thirteen have been taken off the drug for the following reasons:

1. Hematologic relapse—five patients—during the sixth, seventh, eighth, twelfth, and thirteenth months.

2. Neurological relapse—four patients—during the second, sixth, eighth, and twelfth months.

3. Progressive anemia (not real relapse)—one patient—during the thirteenth month.

4. Refusal to take folic acid—one patient—because of "headaches."

5. Unreliability in taking folic acid—one patient.

6. Unreliability of subjective complaints—one patient.

It is concluded from the studies so far performed that 5 mg. folic acid per day is inadequate to prevent clinical, neurological, or hematologic relapses. Studies are now in progress evaluating the efficacy of 10 mg. per day.

40. EXOGENOUS HEMOCHROMATOSIS

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Five female patients, with severe anemias due to miscellaneous causes, who received multiple transfusions and developed hemosiderosis and fibrosis of the liver were studied. The patients ranged in age from 19 to 75 years and were followed from nine months to four and one-half years. They received from twenty-one to seventy-five blood transfusions. With the evidence available from the study of the cases, together with a review of eight similar previously reported cases in the literature, the conclusion is reached that hemochromatosis in man can be experimentally produced by the administration of large amounts of iron (in the form of hemoglobin) if the iron is not secondarily lost from the body.

That the cases were not primary hemochromatosis is evidenced by the fact that the disease occurred in women, many of whom were young, and was accompanied by a severe anemia.

The pathogenesis of both idiopathic (endogenous) hemochromatosis and secondary (exogenous) hemochromatosis is discussed.

With the increased use of blood transfusions and the consequent lengthening of the anemic patient's life, the incidence of exogenous hemochromatosis will increase and will afford an opportunity to study a hitherto poorly understood disease entity.

41. MYELOMA CELL CYTOLOGY AS REVEALED BY HISTOCHEMICAL METHODS*

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(INTRODUCED BY LOUIS R. LIMARZI, M.D.)

There has been no attempt made thus far to relate the cytoplasmic vacuoles, areas of hyaloplasm, and perinuclear clear area of the myeloma cells, as revealed by the Romanowsky stains, to the presence of preformed cytoplasmic organoids. In five cases of multiple myeloma of the "plasma cell" type, sternal marrow and air-dried marrow smears were studied by various cytologic and cytochemical techniques. By these means it has been possible to correlate cytoplasmic structures (mitochondria and portions of the Golgi element) with these cytoplasmic clear areas and, in part, to determine their chemical composition.

Marrow smears stained with Wright's and May-Grünwald-Giemsa stains indicated that these cytoplasmic clear areas (variously described by other authors) exhibited a series of distinctive patterns. Rodlike, filamentous, circular, and dotlike areas of hyaloplasm could be readily delineated. These light areas were found to be either diffusely scattered throughout the cytoplasm or congregated on one side of the nucleus, giving rise to a "Hof" or perinuclear clear zone.

Supravital studies on aspirated sternal marrow, utilizing neutral red and the neutral red-Janus green combination, demonstrated a variety of mitochondria whose form and distribution were identical with the clear areas noted in the stained smears. Neutral red vacuoles were present in all of the myeloma cells; however, they appeared to contribute but a small share to the total cytoplasmic pattern.

*Work done while Research Fellow, Department of Medicine, University of Buffalo School of Medicine, Buffalo, N. Y.

The Bensley-Cowdry mitochondrial technique revealed the presence of numerous mitochondria whose general configuration and distribution within the cells correlated with the observations made on supravital study.

The myeloma cells, when stained with the fat stains sudan black B and sudan IV, exhibited numerous sudanophilic organoids which were identical cytologically with the structures demonstrated by the aforementioned techniques. These bodies were not stained by sudan III, nor did they exhibit a positive Schultz reaction. They were stained by Nile blue sulfate; however, the staining was of the nonspecific type. In view of these negative findings, neutral fats, cholesterol, and cholesterol esters were excluded as being responsible for their sudanophilic properties. The organoids were stained by the Smith-Dietrich method, indicating their phospholipid nature.

The cytochemical test for vitamin A and/or its carotene precursors was positive and was precisely localized to the cytoplasmic organoids under discussion. Thus far it has been impossible to localize vitamin C within this group of cells.

All attempts at the cytochemical localization of the aromatic group of amino acids and the sulfhydryl-containing amino acids within these intracellular structures have been uniformly unsuccessful. A positive reaction for the free alpha amino acid groups has, however, been obtained.

Phase microscopy was used in this study as a method of providing a positive correlation between the mitochondria and neutral red vacuoles and the clear areas in the basophilic spongioplasm.

It is concluded that (1) the areas of hyaloplasm in the myeloma cells are due to the presence of underlying mitochondria and portions of the Golgi element; (2) these structures have been found to be of a phospholipid nature; (3) vitamin A and/or the carotinoid pigments are localized to these areas; (4) these structures give a cytochemical reaction for free alpho amino acid groups.

42. BLOOD AND BONE MARROW FINDINGS IN RENAL DISEASE

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The association of anemia and renal impairment has been recognized for some time. Because the anemia is refractory to treatment and has no positive distinguishing characteristics, it has not been diagnosed in many instances. In addition there has been no agreement as to the hematologic findings.

In this study 102 patients were included, divided into 44 with chronic glomerulonephritis with azotemia, 22 without azotemia, 20 with essential hypertension, 6 with acute glomerulonephritis, 8 with miscellaneous kidney diseases, and 2 with extrarenal uremia. As a result of these studies it has been shown that anemia as demonstrated by the hemoglobin, red blood count, and hematocrit values, in general, is inversely related to the elevation of the nitrogenous waste products in the peripheral blood. The hemoglobin averaged 8.5 Gm. per 100 ml., the red blood count, 2,900,000 per cubic millimeter, and the hematocrit, 25 per cent, in cases of chronic glomerulonephritis, with an average nonprotein nitrogen of 123 mg. per 100 milliliters. The leucocyte count averaged about 12,500 in the cases of acute glomerulonephritis. Chronic types of renal disease showed borderline elevations of the leucocyte count. A slight increase in the relative percentage of stab neutrophiles was also present in these respective cases. The average of the mean corpuscular volume, hemoglobin, and hemoglobin concentration in these studies proved to be within normal

limits for these groups. There were individual variations, but generally they involved the hemoglobin values and not those of the mean corpuscular volume as had been reported by other investigators.

A further correlation of the retained nitrogenous products and the values for the hemoglobin, hematocrit, and red blood count showed that in ten of the twenty-two patients with chronic glomerulonephritis without azotemia a significant variation was noted. Eight of these ten revealed a rise in one or two of the four blood chemistry determinations (nonprotein nitrogen, urea-nitrogen, uric acid, and creatinine).

The bone marrow, in contrast to some of the other published data, revealed a fairly adequate cellular picture with a myeloid-erythroid ratio near the normal in all the groups. The patients with acute and chronic glomerulonephritis with azotemia showed a slight increase in myeloid elements. The relative percentage of polychromatic normoblasts, however, was normal in all groups. The myeloid-erythroid layer, determined following centrifugation of 1 ml. of marrow blood in a hematocrit tube, was not increased in any of the groups except in acute glomerulonephritis. This increase was due to an increase in myeloid elements.

As a result of these studies, the following conclusions can be drawn: (1) anemia in renal disease is inversely related to the levels of nitrogenous waste products in the blood; (2) in cases where the anemia is borderline and renal disease is suspected, not only one, but complete blood chemistry studies should be done serially; (3) the bone marrow is relatively normal with some evidence of myeloid hyperplasia. Hypoplasia or maturation arrest of the bone marrow elements was not observed.

43. A STUDY OF THE MARROW IN ARTHRITIS

EUGENE F. TRAUT, M.D., AND STEVEN O. SCHWARTZ, M.D., CHICAGO, ILL.

Since evidence regarding the nature of arthritis has been sought often in the peripheral blood, it was thought advisable to study the most important blood-building organ, the marrow. We were interested in learning whether the marrow would help distinguish the two great groups of nonspecific chronic arthritis and whether the marrow findings paralleled the clinical progress in these conditions.

Forty arthritic patients were studied: nineteen atrophic, fifteen hypertrophic, two psoriatic, and four patients with rheumatic fever. These patients were in various stages of activity. Two of the patients with atrophic arthritis had 2 marrow studies.

One of us (S. O. S.) studied the marrows and gave a report without access to other clinical findings.

In general, the marrow mirrored the clinical impression gained otherwise. The marrow study gave further evidence of the inflammatory nature of atrophic (rheumatoid) arthritis as compared with hypertrophic arthritis. Complicating infections can cause the myelogram of degenerative joint disease to resemble that of inflammatory arthritis. The changes in the marrow reflect the course of disease in the joints.

The findings in the few patients with rheumatic fever suggest marrow studies as a measure of the activity of the rheumatic process.

44. RESULT OF METHYL-BIS (BETA-CHLOROETHYL) AMINE HYDROCHLORIDE THERAPY: A PRELIMINARY REPORT OF A NITROGEN MUSTARD

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(INTRODUCED BY JAMES A. GREENE, M.D.)

Fourteen patients with confirmed diagnosis of lymphoma, leucemia, and multiple myeloma received single courses of methyl-bis (beta-chloroethyl) amine hydrochloride intravenously in the usual dosage.

Of six patients with Hodgkin's disease, two had an excellent remission and one a satisfactory response, whereas the remaining three improved either minimally or not at all. Nitrogen mustard administration provoked only partial remissions in two cases of lymphosarcoma and minimal improvement in two cases of chronic myelogenous leucemia. One patient with chronic lymphogenous leucemia obtained a rapid decrease in the leucocytosis without other evidence of benefit.

Two patients with severe skeletal pain due to multiple myeloma were improved symptomatically to a striking degree, but roentgen studies failed to demonstrate regression of the bone lesions. A case of mycosis fungoides was unaffected by nitrogen mustard therapy.

Nine patients of the entire series had received no previous treatment, whereas five had been subjected to varying amounts of roentgen therapy. In this limited clinical experience the previous use of irradiation did not appear to affect the response to the nitrogen mustard in an adverse way.

Excluding the three cases of leucemia, a significant leucopenia followed the administration of the drug in five patients (42 per cent of the series), but no ill effects ascribable to the leucopenia were detected. Nausea and vomiting occurred in 64 per cent of these patients during the course of treatment and constituted the only clinical evidence of toxicity in this series.

45. EXPERIENCES WITH URETHANE IN EIGHT PATIENTS WITH MALIGNANT DISEASE

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The therapeutic action and toxic effects of urethane have been studied in eight patients with leucemia and other neoplastic conditions. Oral administration was tolerated by most patients in doses of 0.5 to 6.0 Gm. daily over periods ranging from nine to fifty-four days. Total doses ranged from 26.5 to 221 grams.

No changes were noted in renal or liver function as observed by complete urinalysis, phenolsulphthalein, urea clearance, bromsulphthalein, hippuric acid, and cephalin flocculation tests.

There was little or no clinical improvement in three of four patients with leucemia exhibiting hematologic responsiveness and decrease in size of enlarged lymph nodes, spleen, or liver. Urethane did not affect the progress of terminal subacute leucemia, myelomatosis, or carcinoma of the lung. Although nausea and vomiting occurred frequently, there was no evidence that these symptoms constitute an indication for stopping urethane treatment. Weight loss out of

proportion to reduced food intake was noted in four patients and may represent an important constitutional injury to patients receiving urethane. Transient drowsiness and slight dizziness were observed.

Urethane caused a fall in the absolute numbers of both lymphoid and myeloid leucocytes in the blood of patients with normal or leucemic counts. The effect is greater on the more undifferentiated leucocytes. Severe temporary hypoplasia of all elements of the bone marrow may result from overtreatment and may progress after treatment has been stopped. Megakaryocytogenesis persists longer than erythrocytogenesis or leucogenesis and appears to recover earliest after urethane therapy.

In the chronic leucemias, initial counts between 22,350 and 133,800 per cubic millimeter dropped to levels ranging between 1,900 and 12,000 per cubic millimeter after the patients received from 70 to 116 Gm. of urethane. One patient with chronic myelogenous leucemia exhibited a rise in the platelet count although the leucocyte count dropped to normal. There is no correlation between daily or total dose of urethane and the rate or extent of change of the total leucocyte count, lymph nodes, liver, spleen, or bone marrow.

No significant changes in reticulocyte percentages or color indices were observed. Anemia was favorably influenced in some patients although in others it became more severe.

There was regression of lesions in cases of mycosis fungoides and carcinoma of the skin.

On the basis of our studies it appears necessary to make bone marrow examinations, determinations of absolute leucocyte counts, and estimation of platelets, hemoglobin, and erythrocyte levels frequently.

46. BLOOD COAGULATION DURING INTRAVENOUS INJECTION OF HISTAMINE

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It has been recognized for some time that during peptone and anaphylactic shock and thrombocytopenic purpura there occurs a simultaneous clumping out of platelets and vasodilatation. Theoretically this dilatation occurs because platelets are no longer available for the removal of free histamine from the blood, and this histamine is the cause of the vasodilatation. Some investigators, however, believe that the platelets themselves are a source of some of the body histamine.

We have attempted to discover whether a continuously high level of histamine in the blood for many hours will materially effect the qualitative platelet count either by driving platelets out of the free circulation or by causing their increase in an attempt to overcome the increased histamine concentration.

Fifteen patients who received intravenous injections of histamine for migraine were used. Blood was drawn before the onset of each injection and six hours later, toward the end, using the technique of Sanford and Leslie. Blood was collected in chilled syringes, counted on a chilled chamber, and centrifuged in waxed chilled tubes. The coagulation times for whole blood, cell-free plasma, and platelet-free plasma were determined. Hematocrit and platelet counts were made simultaneously.

The histamine was given at a constant rate of 20 drops per minute in 500 ml. of normal salt solution, but the amount of histamine was almost doubled with each injection, 0.2 mg. being used for the first injection and 2.0 mg. of histamine base for the last.

Results.—

1. There was a definite shortening of the coagulation time in most cases which in total varied from 1.9 minutes to 1.0 minutes.

2. There was a correlation between the qualitative platelet activity and the coagulation time.

47. THE EFFECT OF CHRONIC ANEMIA ON RENAL FUNCTION AS MEASURED BY INULIN AND DIODRAST CLEARANCES

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This study has been made as a means of evaluating renal function quantitatively in patients with chronic anemias. Inulin and diodrast clearances were determined in fourteen patients; a total of twenty separate studies were made.

In five patients with pernicious anemia, nine renal clearance studies were obtained. The average glomerular filtration rate was 73 ml. per minute as compared with a control average of 119 ml. per minute. The average renal plasma flow in the patients with pernicious anemia was 419 ml. per minute (control average, 640 ml. per minute). The average effective renal blood flow in these patients was 617 ml. per minute (control average, 1050 ml. per minute). These results reflect very definite decreases in glomerular filtration rate, renal plasma flow, and effective renal blood flow. However, the most striking abnormality is evident in the effective renal blood flow.

Eight studies in six patients with sickle cell anemia revealed an even greater reduction of glomerular filtration rate, with an average of 60.2 ml. per minute. The average renal plasma flow in this group was reduced to about the same degree as in the patients with pernicious anemia with an average of 398 ml. per minute. The effective renal blood flow was determined in only four instances; hence, the average of 386 ml. per minute is not comparable.

It is significant that the effective renal blood flow was definitely increased in two patients with pernicious anemia after remissions had been induced following the institution of specific therapy.

Chronic anemia may be associated with decreases in glomerular filtration rate, renal plasma flow, and effective renal blood flow. The impairment in glomerular filtration rate is more marked in patients with sickle cell anemia than in patients with pernicious anemia. There is a tendency for recovery of these functions toward normal in patients with pernicious anemia as a remission develops.

48. THE TREATMENT OF EXPERIMENTAL UREMIA BY INTESTINAL LAVAGE

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With the marked interest in peritoneal irrigation as a treatment of uremia, it was felt advisable to explore further the possibilities of intestinal irrigation despite a few unfavorable reports in the literature.

Isolated high intestinal loops (Thiry-Vella fistulas) were prepared in dogs and the intestinal continuity restored by end-to-end anastomosis. Eleven to twenty-eight days later a bilateral nephrectomy was performed. Five control dogs so treated died in from 52 to 104 hours following nephrectomy (average, seventy-three hours) and the terminal blood urea nitrogen levels varied from 145 to 265 mg. per cent (average, 193 mg. per cent). Six experimental dogs so treated, but, in addition, submitted to irrigations of the intestinal loop, died in from sixty-three to ninety-nine hours following nephrectomy (average, eighty-one hours) and the terminal blood urea nitrogen levels varied from 89 to 229 mg. per cent (average, 144 mg. per cent). It is thus seen that the duration of life in the irrigated dogs was not appreciably lengthened, probably because of a severe disturbance in the electrolyte pattern of the blood, which was only slightly relieved by calcium gluconate.

However, the average terminal blood urea nitrogen level was lowered by about 26 per cent. Furthermore, during the eighteen irrigation procedures to which the six experimental dogs were submitted, the blood urea nitrogen level was kept constant or was lowered in eight, or 45 per cent. The amount of urea nitrogen removed varied from 1.3 to 14 mg. per inch of intestine irrigated per hour. Best results in this regard were obtained with hypertonic fluids.

Conclusion.—Irrigation of intestinal loops will remove fairly large amounts of urea but does not appreciably prolong the life of the animals tested.

49. EXPERIENCES WITH THE MURRAY ARTIFICIAL KIDNEY

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At the 1947 meeting of the Central Surgical Association, Gordon Murray of Toronto described an artificial kidney with which he had had some success in the removal of nitrogenous products from anuric dogs and human patients. The principle was that of dialysis through a cellophane membrane. Blood was caused to flow through small-bore cellophane tubing which was immersed in a solution containing the dialyzable substances which it was desired to retain. The blood was removed from one iliac vein by a catheter inserted through the saphenofemoral junction and was allowed to return into the other iliac vein. Propulsion of the blood was obtained by a sterile pump arrangement operated by an electric motor. The dialysate was maintained at body heat and was agitated by a suitable mechanical device. A trap was provided to prevent air embolism.

We prepared an apparatus similar to that of Murray. The dialysate solution was that of Kolff of Holland. It had the following composition: NaCl, 0.6 per cent; $NaHCO_3$, 0.2 per cent; KCl, 0.04 per cent; and glucose, 1.3 per cent. We have used the apparatus on ten nephrectomized dogs and two anuric patients. Approximately 2 Gm. of nonprotein nitrogen were removed each

hour from dogs whose blood nonprotein nitrogen was elevated to 100 to 200 per 100 milliliter. The chief adverse effect was related to the use of heparin, which is necessary to maintain the fluidity of the blood.

The first patient was a man, aged 21 years, with greatly diminished renal function due to repeated episodes of hemolysis. The artificial kidney was attached for eight hours, during which time his blood was dialyzed against 120 liters of solution. The initial nonprotein nitrogen was 260 mg. per cent. Although 27 Gm. of nonprotein nitrogen were recovered, the final nonprotein nitrogen of the blood showed a paradoxic increase to 294 mg. per cent. The run was discontinued because of serious bleeding around one of the catheters. The patient expired in pulmonary edema twelve hours after the apparatus had been disconnected.

The second patient was a man 52 years of age, with acute hepatitis and urinary suppression. He had been comatose for two days. It was questioned if the apparatus might serve as an "artificial liver" as well as an "artificial kidney." The apparatus was operated for twenty-nine and one-half hours; 81.6 Gm. of nitrogen were removed, the blood nonprotein nitrogen falling from 129 to 89 mg. per cent. There was no change in the blood sugar level, the chlorides rose from 365 to 427 mg. per cent, and the calcium reached the very low level of 3.5 mg. per cent. Fifty milliliters of 10 per cent calcium gluconate had been given to combat this calcium loss. At no time did the patient show clinical tetany. He expired eight hours after the run. No autopsy was obtained.

It appears that an apparatus of this type is effective to a limited extent in removing nitrogenous products. There is danger of hypocalcemia if the dialysate formula of Kolff is used.

50. LIPOID NEPHROSIS

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Clinical and biochemical studies of forty-one children with lipoid nephrosis are evaluated. Twenty-six of the children are alive; nineteen have been entirely well for one to sixteen years, including three who had minimal hematuria on various occasions. Three have been well for less than a year and four still have active disease. Of twenty-two children who had received sulfonamides for infections, only three have died. Necropsy was done on ten of the fifteen children who died. Minor glomerular changes were seen in the kidneys of four children not known to have had hematuria. Pronounced glomerular changes occurred in two children who had minimal hematuria. Of the four children who did not show any glomerular changes, two had minimal hematuria on several occasions. Pathologic changes in the liver were minimal unless acacia had been given.

Laboratory determinations revealed no significant alteration of the prothrombin time. Most of the increase in the blood fats was in the cholesterol fraction. The percentage of cholesterol esters was not decreased. In case of recovery, the cholesterol returned to normal values at a slower rate than the serum albumin. Blood fats reached values exceeding 5 Gm. per 100 ml. in some instances. No "critical edema level" for albumin was found. We have seen children free from edema with albumin values below 1 Gm. per 100 ml. and others with edema with values of 2.0 Gm. per 100 ml. The sedimentation test was a reliable guide in determining the course of the disease. Electrophoretic analyses of thirty plasma samples revealed uniformly low albumin values.

while the most pronounced change was the increase of the alpha 2 and beta fractions of globulin. During recovery, albumin increased, but the gamma globulin was slower in attaining normal values than the other fractions. No correlation was found between changes in the sedimentation rate and any individual protein fraction.

Choline citrate (0.8 Gm. each day) given to seven children for prolonged periods failed to produce essential changes in the blood fats or definite clinical improvement.

Periods of sudden change in edema were found to be critical times for the nephrotic child. Two children developed cardiac failure after the injection of large volumes of sucrose.

Early diagnosis is important, for treatment is most effective in the early stage.

The prognosis is good since the advent of chemo- and antibiotic therapy and is not impaired by prolonged duration. Growth of the children who recovered was good.

(To be concluded in the December issue.)